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STUDIES IN THE PHYSICAL CHEMISTRY OF THE PROTEINS.

II. The Relation between the Solubility of Casein and Its Capacity to Combine with Base. The Solubility of Casein in Systems Containing the Protein and Sodium Hydroxide.*

By EDWIN J. COHN† and JESSIE L. HENDRY.

(From the Department of Physical Chemistry in the Laboratories of Physiology, Harvard Medical School, Boston.)

(Received for publication, February 1, 1923.)

I.

INTRODUCTION.

"Casein is known to have the character of a weak acid; it reddens wet litmus paper and forms salt-like compounds with metals."[1] Thus Laqueur and Sackur began their classic paper on casein nearly 20 years ago.[2] Moreover, the relative strength of this acid was suggested even before modern methods were available for its determination, since it "has the power of expelling carbonic anhydride from soluble carbonates and bicarbonates."[3] The amount of base with which it combines at acid, at neutral, and at alkaline reactions has perhaps been investigated more often than any similar reaction

* A report of this investigation was read before the Sixteenth Annual Meeting of the Society of Biological Chemists (1).

† National Research Fellow in Chemistry at the time these researches were begun.

[1] "Das Kasein hat bekanntlich den Charakter einer schwachen Säure: es rötet feuchtes Lackmuspapier und bildet mit den Metallen salzähnliche Verbindungen," Laqueur and Sackur (2), p. 193.

[2] Their survey of the earlier investigations is altogether admirable.

[3] Osborne (3), p. 399.

involving a protein.[4] Nonetheless, it seemed desirable, in connection with an investigation of the relation between the solubility of proteins and the degree of their amphoteric dissociation, once again to study the neutralization of casein by base.

The hypotheses underlying this investigation, and the history of their development, have been explained in detail in a previous communication (17). In substance they are that proteins are amphoteric electrolytes, that they dissociate as acids and as bases, and that the ions due to their dissociation combine respectively with bases and acids. Furthermore, that the compounds of proteins with acids and with bases are for the most part relatively soluble, especially in comparison with uncombined protein. When uncombined, a protein dissociates into anions and cations. If it dissociates into as many anions as cations it is said to be in an isoelectric condition, or at its isoelectric point (18). Finally, that the solubility of proteins in the neighborhood of their isoelectric points depends in large part upon the degree of their dissociation, the more soluble proteins being the more dissociated (19).

Casein, the uncombined protein, is only very slightly soluble. Indeed, Osborne concluded that "caseinogen is either completely insoluble in water or else possesses a solubility so low as not to be measured quantitatively by ordinary methods"[5] and Laqueur and Sackur believed that casein had "no measurable solubility in pure water at room temperature."[6] Casein has, however, a very definite and constant solubility, as we have recently shown, of 0.11 gm. in 1 liter at 25°C. (17). This solubility is, within wide limits, independent of the amount of casein precipitate with which the solution is in heterogeneous equilibrium. Moreover, the casein that dissolves at the isoelectric point must, in the absence of any compounds of casein, be either the casein molecule, or ions formed by dissociation of the casein molecule. The solubility data that have been published thus far furnish no information regarding the ratio of the molecule

[4] Since Laqueur and Sackur, Robertson (4–6), Long (7), Van Slyke and his associates (8–13), Yamakami (14), Clark (15), and Loeb (16) have studied the base-binding capacity of casein.

[5] Osborne (3), p. 398.

[6] Laqueur and Sackur (2), p. 197.

to the dissociated ions of casein; that is, to the degree of dissociation of casein at its isoelectric point. If they did it would be possible, as will appear from a theoretical consideration of the equilibria involved, to calculate the strength with which casein dissociates as an acid and therefore the strength with which it combines with base.

Although so very insoluble in the free state, casein becomes very soluble when combined with base. It therefore seemed possible, in the first place, to determine the amount of protein with which each mol of base combined, that is the composition of the compound or compounds that casein forms with sodium hydroxide; and in the second place, to determine the strength of casein as an acid by measuring the rate of change of the hydrogen ion concentration as the casein was neutralized by base. It was believed that the simultaneous measurement of these two quantities would completely characterize such diphasic systems, and yield both the degree of dissociation of the acid groups in casein and their equivalent combining capacity for base.

II.

The Preparation of Casein.

The measurements of solubility have been made with great accuracy upon very pure casein. The method of preparing casein that we have employed has already been described (17). It is a modification of the method of Van Slyke and Baker (20).

After the casein had been precipitated at its isoelectric point and washed with distilled water according to the method of Van Slyke and Baker, only enough sodium hydroxide was added to bring the casein to a neutral reaction. In this our method differed from that of Hammarsten in which enough sodium hydroxide is added to dissolve the casein completely. Loeb has observed that if a divalent rather than a monovalent base is used to dissolve isoelectric casein, twice the normal concentration is required. As a result the hydroxyl ion concentration of casein dissolved by calcium hydroxide is very much greater than that dissolved by sodium hydroxide. Loeb's observation suggests, therefore, that the casein that persists (undissolved) at a neutral reaction is largely combined with divalent bases. We have accordingly removed and discarded this precipitate, in the later preparations by centrifugation (in a Sharples centrifuge at about 30,000 revolutions a minute), and then by filtration through filter paper pulp. The casein in the filtrate of the centrifugate was then reprecipitated at its isoelectric point by the addition of hydrochloric acid, washed, and again dissolved by sodium hydroxide.

It has been our experience that the casein completely dissolved the second time at a neutral reaction. The casein in this clear neutral solution was finally precipitated at the isoelectric point and purified.

The acid used was usually hydrochloric, and was delivered very slowly from a capillary tip extending well into the solution. The solution was continuously and rapidly mixed by a motor-driven glass screw-shaped stirrer, which constantly forced fresh portions of the protein past the glass tip from which the acid was being delivered, in a manner similar to that described by Van Slyke and Baker. In this way the protein was never exposed to the denaturing effect of a local excess of acid. After precipitation had begun, the process of neutralization was further retarded in order to allow new states of equilibrium to be fully attained. As the end was approached, small samples were removed and the hydrogen ion concentration was electrometrically determined.

Stirring was always continued for many hours after the isoelectric point was reached, in part to break up the flocks or aggregates that form under these conditions and to prevent the occlusion of impurities in them, in part to hasten the attainment of equilibrium in so sluggish a system. The protein was finally returned to the cold room, from which it was never removed for more than a few hours, and the precipitate was allowed to settle. The water-clear supernatant liquid was then decanted, distilled water was added approximately to the same volume, and the precipitate was again stirred for several hours. This operation was usually repeated from six to fifteen times, and was only stopped when the wash water no longer contained chloride, and when, as in Preparation V, the amount of protein dissolving in the wash water had become constant.

When the isoelectric protein had finally ceased to give off impurities and readily soluble protein, it was suspended in the desired volume, and aliquot parts used for analyses. It has been found impracticable to dry proteins, since it is almost impossible uniformly to wet their surface after they have been dried by alcohol and ether. As a result, there can be no guarantee that a true equilibrium is subsequently reached between all of the protein and its solvent. Moreover, it has been thought preferable, for the purposes of this investigation, to retain any lipoid that might be in fixed combination with our proteins, rather than risk the danger of denaturing them by alcohol or ether.

Three considerations indicate the purity of the casein preparations that were employed in this investigation. In the first place, their ash content was invariably low, but was very much lower in the later than in the earlier preparations. The method of Sharples centrifugation was resorted to only in the last three preparations, and has proven a most effective means of reducing the content of ash, and probably of calcium, as the following table shows. The ash contents of our preparations and of those of Van Slyke and Baker (20) were almost identical.

In the second place, Preparations III and V were also used in determining the solubility of uncombined casein. These experiments have already been reported. The results that they yielded could not have been obtained had another protein been present (unless it had the same isoelectric point and the same solubility as casein), or had a compound of casein been present.

TABLE I.

Ash of Casein Preparations.

Preparation.	Casein.	Ash.	Ash in casein.
	gm.	*gm.*	*per cent*
I	1.1081	0.0046	0.41
III	1.0793	0.0043	0.40
	0.9813	0.0039	0.40
IV	0.2238	0.0010	0.45
	0.2469	0.0007	0.28
V	0.3958	0.0004	0.11
	0.7156	0.0006	0.09
	0.7114	0.0006	0.09
VI	1.2489	0.0011	0.09
	1.1428	0.0010	0.09
	1.2696	0.0018	0.14
VII	0.7228	0.0011	0.15

In the third place, the results obtained when the different preparations of casein were dissolved by the same amount of base, in the experiments that are now reported, are so closely concordant as to suggest the identity of the protein in the different preparations.

III.

The Measurement of Solubility.

The methods of measuring the solubility of proteins or of protein compounds that we have employed have also previously been described in great detail (17). They are only summarized here.

Since our casein preparations existed as flocculent precipitates at their isoelectric points, it was necessary to construct a shaking machine by means of which the protein precipitate could be maintained in a state of fine subdivision during pipetting. With the aid of such an apparatus we have been able to sample protein suspensions with an accuracy of 0.3 per cent.

After the protein had been delivered into the volumetric flasks, the solvent was slowly added, drop by drop, from a burette until it rose to the graduated mark on the neck of the bottle. A drop of toluene was added to prevent bacterial action. The flasks were finally stoppered, fixed in place in the carriage of a specially designed shaking machine, and shaken in a water thermostat at 25.0° ± 0.1°C.

In our experience the heavy flocculent protein has usually been brought into equilibrium with its solvent in from 24 to 72 hours. The shaking machine was then stopped, the contents of the flasks were allowed to settle slightly, and the saturated solutions were filtered on No. 42 Whatman filter papers. By means of a water jacket through which the water of the thermostat was kept in constant circulation by a rotary pump, the filtration was carried on at nearly the same temperature as the bath. At least 8 cc. of the protein solution were always used to wash the filter paper, and were accordingly discarded. Aliquot parts of the remainder of the filter were measured out with carefully calibrated pipettes into Kjeldahl flasks. Analyses were usually made in triplicate.

IV.

The Solubility of Casein in Systems Containing the Protein and Sodium Hydroxide.

The experiments upon the effect of sodium hydroxide upon the solubility of casein have involved seven different casein preparations and have extended over 3 years. In them the amount of casein has been varied from 0.5 to 2 gm. in 100 cc., and the amount of sodium hydroxide from 0.5 to 40 $\times$ 10^{-5} mols. That is to say, we have extended the series in one direction until the casein dissolved by the small amount of base was of the same order as the amount that is soluble near the isoelectric point, and in the other, until certain complicating phenomena made it impossible to obtain reproducible measurements.[7]

[7] Yamakami (14) has shown that under certain circumstances salts appreciably increase the solubility of casein. The nature of the effect of salt in dissolving casein is being quantitatively investigated and will subsequently be reported. For the present all measurements of solubilty from the range in which casein behaves most like a globulin have been omitted.

The results that we have obtained are recorded in Table II. The solubility measurements were usually made in triplicate,[8] and the same amount of sodium hydroxide was often added to two or more casein preparations. It will be noted that when but small amounts of sodium hydroxide had been added to the casein, the differences between the different preparations were far greater than the experimental error. Thus Preparation V was consistently less soluble, and Preparation VI consistently more soluble than Preparations III or VII, until from 8 to 10 $\times$ 10^{-5} mols of sodium hydroxide per gm. of casein had been added. Even under these circumstances, however, the results obtained with Preparations III and VII were in close agreement with each other and with the average of Preparations V and VI. We believe that the variations in the last two were due to the same sources of error that were encountered in the measurement of solubility of isoelectric proteins.[9] Chief among these is the difficulty of precipitating the casein in exactly the same physical state, and therefore of removing the last traces of its soluble compounds with equal ease. Consistent with this view is the fact that as the amount of casein dissolved by the sodium hydroxide increased, the variation between preparations became smaller than the experimental error. The solubility due to the casein had become negligible in comparison with the solubility of its sodium compounds.

In our experiments the alkali was usually added to three different amounts of casein in such a way that the ratio of protein to base remained constant. This ratio, as we and others have shown (21–23), determines the hydrogen ion concentration, except for a small variation with concentration, due to depression of the dissociation of the protein compounds. In Table II, therefore, the ratio of sodium hydroxide to casein (mols NaOH added to 1 gm. of casein) is given in Column 1, and the approximate hydrogen ion concentration in Column 2, while Columns 4, 5, and 6 contain the solubility data when casein was present in 0.5, 1, and 2 per cent respectively. In this way isohydrionic comparison of the effect of dilution upon the solubility of the compound or compounds that casein forms with sodium hydroxide is facilitated.

[8] From 5 to 25 cc. were taken for analysis according to the amount of nitrogen contained in the filtrate.

[9] Cohn (17), pp. 715–719.

TABLE II.

The Solubility of Casein in Systems Containing the Protein and Sodium Hydroxide.

NaOH added to 1 gm. casein.	Hydrogen ion concentration.	Casein preparation.	Experiment No.	Protein N in 100 cc. Protein N dissolved in 10 cc. 80 mg.	160 mg.	320 mg.
mols × 10^{-5}	$N \times 10^{-6}$			*mg.*	*mg.*	*mg.*
1.0		III	29	0.28		0.65
				0.28		0.65
				0.28		0.70
		V	40	[0.18	0.35	0.65
				0.20	0.35	0.59
				0.20]	0.32	0.60
		VI	41	0.28	0.50	1.00
				0.28	0.50	0.98
				0.28	0.50	0.98
		VII	42	0.30	0.45	0.73
				0.30	0.43	0.74
				0.30	0.44	0.73
		Average.		0.29	0.43	0.75
2.0		V	40	0.40	0.62	1.20
				0.37	0.63	1.15
					0.63	1.24
		VI	41	0.46	0.80	1.50
				0.46	0.84	1.48
				0.46	0.85	1.50
		Average.		0.43	0.73	1.34
3.0		III	29	0.54		1.88
				0.56		1.87
				0.54		1.85
		VII	42	0.59	1.00	1.90
				0.60	0.98	1.94
				0.59	0.97	1.90
		Average.		0.57	0.98	1.89
4.0		V	40	0.64	[1.05	[3.20
				0.65	1.00	3.22]
				0.70	1.02]	
		VI	41	0.84	[1.56	[3.62
				0.82	1.56	3.68
				0.82	1.60]	3.62]
		Average.		0.74		

TABLE II—*Concluded.*

NaOH added to 1 gm. casein.	Hydrogen ion concentration.	Casein preparation.	Experiment No.	Protein N in 100 cc. 80 mg. 160 mg. 320 mg. Protein N dissolved in 10 cc.		
mols $\times 10^{-5}$	$N \times 10^{-5}$			*mg.*	*mg.*	*mg.*
5.0		I	6	[0.76 0.76 0.82]	[1.36 1.36 1.33]	[2.40 2.40]
		IV	25	[0.78 0.78 0.79]	[1.37 1.38 1.37]	[2.65 2.69 2.65]
		VII	42	0.92 0.92 0.88	1.70 1.69 1.80	[3.80 3.72 3.77]
		Average.		0.91	1.73	
8.0		V	40	1.40 1.41 1.41	2.90 2.93	5.98 5.98 5.98
		VI	41	1.34 1.28 1.27	2.95 3.00 2.95	[7.02 7.14 7.00]
		Average.		1.35	2.95	5.98
10.0	0.105	IV	24	1.72 1.75 1.73	3.33 3.37 3.56	6.41 6.44 6.66
		VI	46	1.88 1.84 1.73	3.35 3.30 3.29	[5.98 5.94 6.02]
		Average.		1.77	3.37	6.50
15.0	0.067	I	5	2.56 2.52 2.44 2.47	4.87 4.92 5.45 5.63	10.15 10.24
		Average.		2.51	5.22	10.20
20.0	0.050	IV	24	3.37 3.30 3.37	6.67 6.75	13.23 13.26
		Average.		3.35	6.71	13.25

Such a comparison yields, as a first approximation, the result that the amount of casein carried into solution is proportional to the amount of sodium hydroxide added. Solubility, when once equilibrium had been established, was nearly independent, over a considerable range, of the amount of casein precipitate. Whether

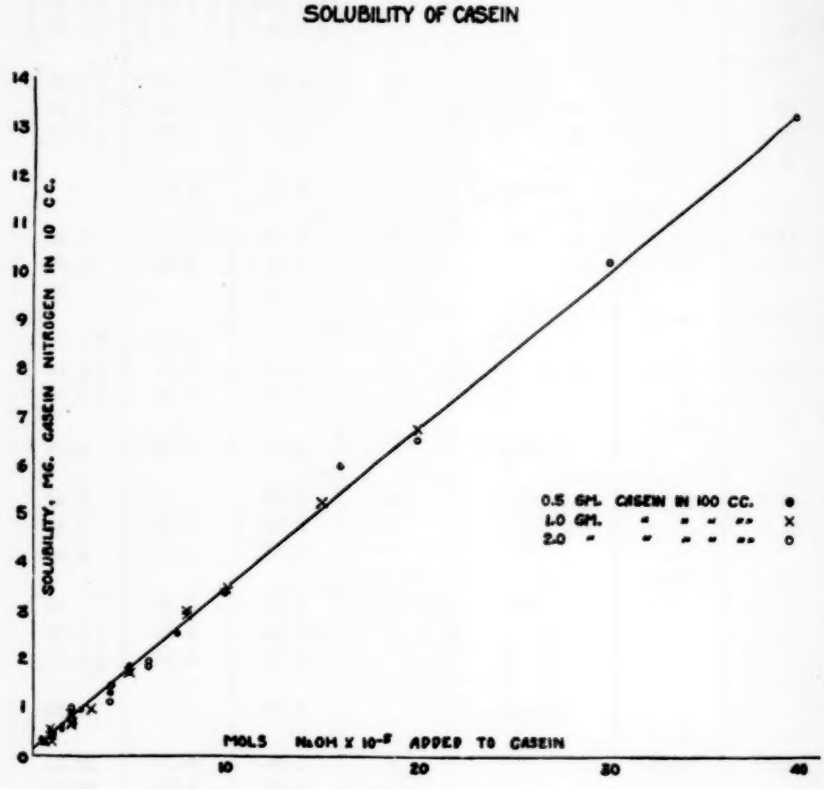

FIG. 1.

sodium hydroxide had been added to 0.5 or to 2 gm. of casein, the amount that dissolved was nearly the same. Moreover, when twice the amount of sodium hydroxide was added, twice as much casein dissolved. In Fig. 1 the abscissæ represent the amount of sodium hydroxide in the systems, the ordinates the amount of casein in solution. With certain exceptions, more common the more concentrated

the casein, the measurements of solubility fall on a straight line, indicating a remarkably constant combining capacity of casein for base.

The weight of casein in combination with each molecule of sodium hydroxide could be calculated from these data, provided all of the casein in solution was combined with base. This calculation has been attempted in Table III, which is arranged in the same manner as Table II. The amount of sodium hydroxide that had been added to each gram of casein is given in Column 1, and in Columns 2, 3, and 4, the averages of the solubilities of the different preparations. These

TABLE III.

Equivalent Combining Ratio of Casein and Sodium Hydroxide Calculated from Solubility Data on the Assumption That the Solubility of the Casein Molecule Is Negligible.

NaOH added to 1 gm. casein.	Protein N in 100 cc.			Protein N in 100 cc.		
	80 *mg.*	160 *mg.*	320 *mg.*	80 *mg.*	160 *mg.*	320 *mg.*
	Protein N dissolved in 10 cc.			Protein N dissolved by 1×10^{-4} mols NaOH.		
mols $\times 10^{-4}$	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
1.0	0.29	0.43	0.75	5.80	4.30	3.75
2.0	0.43	0.73	1.34	4.30	3.65	3.35
3.0	0.57	0.98	1.89	3.80	3.27	3.15
4.0	0.74			3.70		
5.0	0.91	1.73		3.64	3.46	
8.0	1.35	2.95	5.98	3.38	3.69	3.74
10.0	1.77	3.37	6.50	3.54	3.37	3.25
15.0	2.51	5.22	10.20	3.35	3.48	3.40
20.0	3.35	6.71	13.25	3.35	3.36	3.31

have been divided by the sodium hydroxide concentrations in the respective systems, and the quotients are recorded in Columns 5, 6, and 7. The quotients are approximately constant, only when appreciable amounts of casein were dissolved. In the systems containing the smaller amounts of sodium hydroxide, however, the solubility was always greater than could be accounted for merely on the basis of a constant combining capacity of casein for base. There the solubility of the casein itself was no longer negligible in comparison with the solubility of the sodium compound, and a correction for the solubility of the casein had therefore to be found and applied before the data

could be used to calculate the combining weight of casein and sodium hydroxide.

The solubility of the casein molecule can be estimated most accurately in the systems containing the smallest amounts of sodium hydroxide. Only in them, as we have seen, was this component of the solubility larger than the experimental error. Accordingly, the average solubilities in the systems containing the six smallest amounts of sodium hydroxide have been collected and rearranged in Table IV. The concentrations of the sodium hydroxide, rather than the ratio of casein to sodium hydroxide, are given in Column 1, since homogeneous

TABLE IV.

Solubility of Casein in Systems Containing the Protein and Small Amounts of Sodium Hydroxide.

NaOH added to casein.	Hydrogen ion concentration.	Protein N in 100 cc. 80 *mg.*	160 *mg.*	320 *mg.*	Average protein N dissolved in 10 cc.
		Protein N dissolved in 10 cc.			
$N \times 10^{-4}$	$N \times 10^{-5}$	*mg.*	*mg.*	*mg.*	*mg.*
0.5	0.50	0.29			0.29
1.0	0.34	0.43	0.43		0.43
1.5	0.28	0.57			0.57
2.0	0.24	0.74	0.73	0.75	0.74
2.5	0.21	0.91			0.91
3.0	0.20		0.98		0.98
4.0	0.17	1.35		1.34	1.34
5.0	0.15	1.77	1.73		1.75
6.0	0.14			1.89	1.89

buffering in casein solutions is inappreciable until the casein ion formed by dissociation of sodium caseinate is present in greater concentration than in most of these cases.[10] The estimated hydrogen ion concentration, given in Column 2, is therefore determined, in large part, by the concentration of base. The amount of casein dissolved in systems containing the same amount of base, in Columns 3, 4, and 5, have been averaged together in Column 6, regardless of the amount of casein precipitate that they contained. The very im-

[10] This ion again dissociates and the ratio between the concentration of the ions of the successive dissociations then controls the hydrogen ion concentration.

portant observation, that the amount of precipitate in such systems effects the solubility and the hydrogen ion concentration but slightly, must be considered additional evidence that true heterogeneous equilibria have been attained between the precipitated and dissolved casein.

The average data in Table IV are graphically represented in Fig. 2. The abscissæ again represent the amount of base added to the casein, and the ordinates the amount of casein dissolved. Within the errors

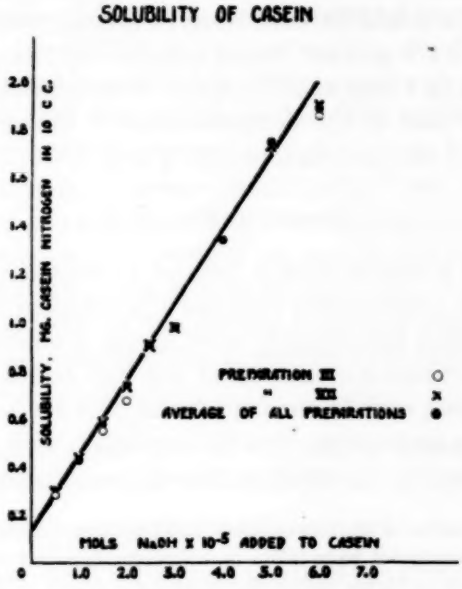

FIG. 2.

of measurement, the points appear to fall on a straight line, in this range also. Accordingly, one must conclude that in this range each increment of base carried an equivalent amount of casein into solution. If no base had been added, however, it would appear that a certain amount of casein would nonetheless have been held in solution, an amount that was also dissolved in the systems containing sodium hydroxide, but was not combined with it. This amount is indicated by extending the straight line connecting the solubility determinations

in systems containing small amounts of sodium hydroxide.[11] Where the abscissa has the value 0, the ordinate has the value 0.14 ± 0.01 mg. of casein nitrogen in 10 cc., or 14 mg. of casein nitrogen in 1 liter at 25°C. The significance of this value appears from the following theoretical considerations.

V.

The Solubility Product Constant.

Experiments that we have previously reported show that casein has a measurable solubility, even when no sodium hydroxide has been added to it, of 0.178 ± 0.006 mg. of nitrogen in 10 cc., or 17.8 ± 0.6 mg. of nitrogen in 1 liter at 25°C. (17). This solubility (S) has been considered the sum of the concentrations of the protein molecule (HPOH) and of the protein ions (HP^+) and (POH^-) into which it dissociates

$$S = (HPOH) + (HP^+) + (POH^-) \quad (1)$$

Equation (1) is written in such form as to suggest that casein dissociates both as a monovalent acid and a monovalent base. In point of fact, as we shall subsequently show, casein combines with base as though it were at least a divalent acid, and with acid as though it were a tetravalent base, even in the neighborhood of its isoelectric point. The simplifying assumption, that in comparison with the concentration of ions formed by the stronger dissociation the ions formed by the

[11] If the concentration of the casein cation had been appreciable in these systems, the lower points would not have fallen on a straight line, but rather on a curve, cutting the ordinate at higher values the greater the degree of dissociation of the protein. In such a case the solubility of the molecule could only be estimated by extrapolation. It is perhaps important to note that if the curves describing proteins passing through minima of solubility in the neighborhood of their isoelectric points were sufficiently accurate they could be analyzed in terms of these three components. A limiting case occurs, on the one hand, when the solubility of the protein is immeasurably small. In this case the "curve" becomes two diverging straight lines, originating at the isoelectric point. On the other hand, when the protein is largely dissociated and completely soluble the "curve" becomes a horizontal straight line. Within the triangle enclosed by these three sides the solubility determinations of all proteins must fall. We propose in forthcoming communications to analyze in this manner the series of slightly soluble proteins that we have investigated.

weaker dissociation are negligible, is not adequate to explain our results. For although the concentration of ions formed by the first dissociation of casein as an acid must be much greater[12] than the concentration of ions formed by the second dissociation, our experiments indicate that casein passed into solution as a disodium compound.

Our criteria for this conclusion are twofold. In the first place, as we have already seen, the increase in the solubility of casein was proportional to the amount of sodium hydroxide added to it. If casein had dissolved first as a monovalent sodium compound, and only at lower hydrogen ion concentrations as a disodium compound, this would not have been the case. The combining ratio would have been greater at first than subsequently. The solubility determinations in systems containing casein and sodium hydroxide would therefore not have fallen upon a single straight line.

In the second place, if casein had dissolved as a monosodium compound the change in the hydrogen ion concentration brought about by the addition to casein of sodium hydroxide would have been inversely proportional to the sodium hydroxide concentration. In that case the dissociation could be defined by the mass law equation

$$(H^+)\,(HPOH^-) = Ka_1 \cdot (H_2POH) \qquad (2)$$

Our results indicate that the ion $(HPOH^-)$ formed by the first acid dissociation of casein again dissociated according to the equation

$$(H^+)\,(POH^{=}) = Ka_2 \cdot (HPOH^-) \qquad (3)$$

Multiplication of these two equations yields the dissociation of casein as a divalent acid

$$(H^+)^2\,(POH^{=}) = Ka_1 \cdot Ka_2 \cdot (H_2POH) \qquad (4)$$

In our experiments the hydrogen ion concentration has changed inversely as the square of even the very smallest amounts of sodium hydroxide that we have added to casein.[13]

[12] Great enough, indeed, to produce a hydrogen ion concentration in water of approximately 1.3×10^{-5} N.

[13] The hydrogen ion concentrations in systems containing casein and sodium hydroxide will be critically considered in a forthcoming communication and the strength of the dissociation constants evaluated from them.

We are for the moment interested in the dissociation of casein as an acid and its combination with base. The assumption may therefore be made that the weak basic dissociation of casein is negligible in systems containing sodium hydroxide. Under these circumstances equation (1) should assume the form

$$S = (H_2POH) + (POH^{=}) \tag{5}$$

and if we define the solubility due to the formation of disodium caseinate in terms of the dissociation of casein as a divalent acid

$$(POH^{=}) = \frac{Ka_1 \cdot Ka_2 (H_2POH)}{(H^+)^2} \tag{4}$$

and the solubility of the protein molecule (H_2POH) as Su, equation (5) may be written

$$S = Su + \frac{Su \cdot Ka_1 \cdot Ka_2}{(H^+)^2} \tag{6}$$

In this equation the solubility of a protein is expressed in terms of the solubility of its uncombined molecule, Su; the constants of its acid dissociations, Ka_1 and Ka_2; and the hydrogen ion concentration. Of these the only variables are the solubility of the casein and the hydrogen ion concentration. Both of these quantities have been determined in the systems containing casein and different amounts of sodium hydroxide (Table IV). For any one system equation (6) may be written

$$S_1 = Su_1 + \frac{Su_1 \cdot Ka_1 \cdot Ka_2}{(H_1^+)^2} \tag{7}$$

and for any other system

$$S_2 = Su_2 + \frac{Su_2 \cdot Ka_1 \cdot Ka_2}{(H_2^+)^2} \tag{8}$$

If the casein molecule has a constant solubility

$$Su_1 = Su_2$$

equations (7) and (8) can be combined

$$S_1 - \frac{Su \cdot Ka_1 \cdot Ka_2}{(H_1^+)^2} = S_2 - \frac{Su \cdot Ka_1 \cdot Ka_2}{(H_2^+)^2} \tag{9}$$

and transposed into the form

$$Su.Ka_1.Ka_2 = \frac{S_1 - S_2}{\frac{1}{(H_1^+)^2} - \frac{1}{(H_2^+)^2}} \quad (10)$$

In this equation, $Su.Ka_1.Ka_2$ represents the solubility product. The solubility product should be constant if the assumptions that have been made in the derivation of the equation have been justified.

These equations are very similar to those that were developed by Bruner and Zawadzki (24) in their studies of the conditions for the precipitation of the metal compounds of hydrogen sulfide in quantitative analysis. They are also very similar to those developed by Harkins and other investigators of the solubility relations between electrolytes. Their studies of quite different systems than those to which the solubility product has been here applied have been summarized by Lewis (25) and by Kraus (26).

For the calculation of the solubility product constant of casein, equation (10) and the values in Table IV of the hydrogen ion concentration and of the solubility in systems containing very small amounts of sodium hydroxide have been used. Each of the nine pairs of values has been equated with each of the others. The results of these 72 simultaneous equations are remarkably constant. They are recorded in Column 4 of Table V.

The average of all the calculations of the solubility product yields the constant

$$K = Su.Ka_1.Ka_2 = 3.4 \times 10^{-12} \text{ mg. nitrogen in 10 cc. at 25°C.}$$
$$= 340 \times 10^{-12} \text{ mg. nitrogen in 1 liter at 25°C.}$$

VI.

The Solubility of the Casein Molecule Calculated from the Solubility Product.

We have found that the product of the solubility of the casein molecule, Su, and its dissociation constants yields a constant. The solubility of the casein molecule in these systems must therefore also have been approximately constant. Substituting the value for the solubility product in equation (6) and transposing we have the relation

$$Su = S_1 - \frac{3.4 \text{ mg. N} \times 10^{-12}}{(H^+)^2} \quad (11)$$

TABLE V.

Solubility of the Casein Molecule Calculated from the Solubility and the Hydrogen Ion Concentration of Systems Containing Casein and Sodium Hydroxide.

$$S = Su + \frac{Su \cdot Ka_1 \cdot Ka_2}{(H^+)^2}$$

Equation.	Solubility.	Hydrogen ion concentration.	Solubility product constant.	Solubility due to combined casein.		Solubility of the casein molecule.	
				Calculated from values of solubility product constant.	Calculated from average solubility product constant.	Calculated from values of solubility product constant.	Calculated from average solubility product constant.
	S	(H+)	$Su \cdot Ka_1 \cdot Ka_2$	$\frac{Su \cdot Ka_1 \cdot Ka_2}{(H^+)^2}$		Su	
	N in 10 cc.	$\times 10^{-8}$	N in 10 cc. $\times 10^{-12}$	N in 10 cc.	N in 10 cc.	N in 10 cc.	N in 10 cc.
	mg.		*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
1 and 2			3.01				
1 " 3			3.19				
1 " 4			3.36				
1 " 5			3.31				
1 " 6			2.91				
1 " 7			3.43				
1 " 8			3.60				
1 " 9			3.40				
1	0.29	5.0	3.27	0.131	0.136	0.159	0.154
2 and 1			3.01				
2 " 3			3.40				
2 " 4			3.55				
2 " 5			3.42				
2 " 6			2.88				
2 " 7			3.50				
2 " 8			3.68				
2 " 9			3.44				
2	0.43	3.4	3.36	0.290	0.293	0.140	0.137
3 and 1			3.19				
3 " 2			3.40				
3 " 4			3.69				
3 " 5			3.42				
3 " 6			2.74				
3 " 7			3.52				
3 " 8			3.72				
3 " 9			3.44				
3	0.57	2.8	3.39	0.432	0.432	0.138	0.138

TABLE V—*Continued.*

Equation.	Solubility.	Hydrogen ion concentration.	Solubility product constant.	Solubility due to combined casein.		Solubility of the casein molecule.	
				Calculated from values of solubility product constant.	Calculated from average solubility product constant.	Calculated from values of solubility product constant.	Calculated from average solubility product constant.
	S	(H+)	$Su \cdot Ka_1 \cdot Ka_2$	$\frac{Su \cdot Ka_1 \cdot Ka_2}{(H+)^2}$		Su	
	N in 10 cc.	$\times 10^{-8}$	N in 10 cc. $\times 10^{-12}$	N in 10 cc.	N in 10 cc.	N in 10 cc.	N in 10 cc.
	mg.		*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
4 and 1			3.36				
4 " 2			3.55				
4 " 3			3.69				
4 " 5			3.19				
4 " 6			2.32				
4 " 7			3.48				
4 " 8			3.72				
4 " 9			3.41				
4	0.74	2.4	3.34	0.580	0.588	0.160	0.152
5 and 1			3.31				
5 " 2			3.42				
5 " 3			3.42				
5 " 4			3.19				
5 " 6			1.39				
5 " 7			3.60				
5 " 8			3.85				
5 " 9			3.45				
5	0.91	2.1	3.20	0.726	0.769	0.184	0.141
6 and 1			2.91				
6 " 2			2.88				
6 " 3			2.74				
6 " 4			2.32				
6 " 5			1.39				
6 " 7			5.21				
6 " 8			4.59				
6 „ 9			3.90				
6	0.98	1.9	3.24	0.897	0.939	0.083	0.041

TABLE V—*Concluded.*

Equation.	Solubility.	Hydrogen ion concentration.	Solubility product constant.	Solubility due to combined casein.		Solubility of the casein molecule.	
				Calculated from values of solubility product constant.	Calculated from average solubility product constant.	Calculated from values of solubility product constant.	Calculated from average solubility product constant.
	S	(H+)	$Su \cdot Ka_1 \cdot Ka_2$	$\frac{Su \cdot Ka_1 \cdot Ka_2}{(H+)^2}$		Su	
	N in 10 cc.	$\times 10^{-6}$	N in 10 cc. $\times 10^{-12}$	N in 10 cc.	N in 10 cc.	N in 10 cc.	N in 10 cc.
	mg.		*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
7 and 1			3.43				
7 " 2			3.50				
7 " 3			3.52				
7 " 4			3.48				
7 " 5			3.60				
7 " 6			5.21				
7 " 8			4.16				
7 " 9			3.34				
7	1.34	1.7	3.78	1.308	1.173	0.032	0.167
8 and 1			3.60				
8 " 2			3.68				
8 " 3			3.72				
8 " 4			3.72				
8 " 5			3.85				
8 " 6			4.59				
8 " 7			4.16				
8 " 9			2.12				
8	1.75	1.5	3.68	1.635	1.507	0.115	0.243
9 and 1			3.40				
9 " 2			3.44				
9 " 3			3.44				
9 " 4			3.41				
9 " 5			3.45				
9 " 6			3.90				
9 " 7			3.34				
9 " 8			2.12				
9	1.89	1.4	3.31	1.688	1.729	0.202	0.161
Averages.			3.39			0.135	0.148

The solubility of the casein molecule, Su, has been calculated in this way, and is reported in Columns 7 and 8 of Table V. In Column 7 the solubility product calculated for that particular system was used to estimate the solubility of the molecule; in Column 8 the average solubility product constant was used.

The solubility of the casein molecule cannot be estimated as exactly as the solubility product constant. There were, it is true, wide deviations in the individual determinations of the latter, especially when the solubilities in the two systems were nearly equal, and when the solubilities were high. But whereas these discrepancies were eliminated in that calculation, they are accentuated in estimating the solubility of the molecule.

Nonetheless the results of the calculation are in good agreement in the systems containing the smaller amounts of dissolved casein. Their variation suggests that the solubility of the casein molecule might have decreased slightly with increase in total solubility. Their average is also in good agreement with the average of all the systems, however calculated, and indicates that the solubility of the casein molecule was approximately equal to 0.14 mg. of nitrogen in 10 cc.

VII.

The Solubility of the Casein Molecule Calculated from the Equivalent Combining Capacity of Casein for Base.

The solubility of the casein molecule can also be calculated from the solubility in the different systems and their sodium hydroxide concentrations, if the assumption be made that the concentration of the casein anion dissociating from the casein molecule was negligible in comparison with that dissociating from the sodium caseinate. The concentration of the compound of casein and sodium hydroxide can in that case be substituted for the casein anion (POH^-) in equation (5). The latter may be expressed as the product of the ratio in which the casein combines with base, Sna, and the sodium hydroxide concentration. For any one system this equation takes the form

$$S_1 = Su + Sna_1 (NaOH)_1 \qquad (12)$$

and for any other system

$$S_2 = Su + Sna_2 (NaOH)_2 \qquad (13)$$

Moreover, if the casein has a constant combining capacity for base

$$Sna_1 = Sna_2 \tag{14}$$

Equations (12) and (13) can in that case be combined

$$\frac{S_1 - Su}{(NaOH)_1} = \frac{S_2 - Su}{(NaOH)_2} \tag{15}$$

and transposed into a more convenient form for the evaluation of the solubility of the casein molecule

$$Su = \frac{S_2(NaOH)_1 - S_1(NaOH)_2}{(NaOH)_1 - (NaOH)_2} \tag{16}$$

The solubility of the casein molecule has also been calculated in this way (Table VI). The four smallest solubilities in Table IV, which are probably known most accurately, have been equated against each other and against the other values in Table IV.

TABLE VI.

The Solubility of the Casein Molecule Calculated from the Solubility and the Sodium Hydroxide Concentration.

NaOH in 100 cc.	Casein solubility.	Mols NaOH × 10^{-5} in 100 cc.			
		0.5	1.0	1.5	2.0
		Observed solubility of casein; mg. N.			
		0.29	0.43	0.57	0.74
		Calculated solubility of the casein molecule.			
mols × 10^{-5}	*mg. N in 10 cc.*	*mg. N in 10 cc.*			
0.5	0.29		0.15	0.15	0.14
1.0	0.43	0.15		0.15	0.12
1.5	0.57	0.15	0.15		0.06
2.0	0.74	0.14	0.12	0.06	
2.5	0.91	0.13	0.11	0.06	0.06
3.0	0.98	0.15	0.15	0.16	0.26
4.0	1.34	0.14	0.13	0.11	0.18
5.0	1.75	0.13	0.10	0.07	0.07
6.0	1.89	0.14	0.13	0.13	0.16
Average.		0.14	0.13	0.11	0.13

Whether we use the relation that obtains between the solubility of casein and the hydrogen ion concentration, or between solubility

and the sodium hydroxide concentration, to estimate the solubility of the casein molecule, the results are very similar. The latter also suggest that the solubility of the casein molecule may not have been rigorously constant, but may have decreased slightly with increase in total solubility. The average solubility of the casein molecule calculated in this manner was 0.13 mg. of nitrogen in 10 cc. This is in good agreement with the results of the other calculation and with the value indicated by extrapolation of the straight line connecting the solubility data in Fig. 2.

VIII.

The Equivalent Combining Capacity of Casein for Base.

Whereas the most accurate data to use in estimating the solubility of the casein molecule are clearly obtained from the systems containing the smallest amount of base, the most accurate data to use in estimating the equivalent combining capacity of casein for base are the systems containing larger amounts. In them, as we have seen, the correction to be applied for the casein molecule becomes smaller than the experimental error, as does the variation from preparation to preparation. No large error would be involved, therefore, if the straight line drawn through all the experimental points in Fig. 1 were used as the basis for the calculations.

Although so nearly negligible in this range, the correction becomes significant at lower sodium hydroxide concentrations. In Table III we divided the solubility determinations by the sodium hydroxide concentrations without making any correction for the solubility of the undissociated casein molecule, and the quotients were only constant in the systems that contained large amounts of sodium hydroxide. In the last section we concluded that the solubility of the casein molecule was approximately 0.13 mg. of nitrogen in 10 cc. We are therefore in a position to subtract this amount from the determined solubilities, and divide the remainder by the sodium hydroxide concentration on the assumption that it represents combined casein.

The results are confirmatory evidence of the magnitude of the concentration of the casein molecule. The equivalent combining ratio of casein for base becomes very much more constant when this correction is applied, especially in the systems where the concentra-

tion of the casein molecules was of the same order as the concentration of the casein compounds.

However, it should be noted that an even more constant combining ratio is obtained if a slightly smaller solubility is assumed for the casein molecule. The correction appears to be larger than would be necessary to yield a constant combining ratio. Whether or not this indicates that one or another of the simplifying assumptions that have been made in its calculation requires revision must await further experimentation. Most probably the solubility of the casein molecule

TABLE VII.

Equivalent Combining Ratio of Casein and Sodium Hydroxide Calculated from Solubility Data.

Allowing 0.13 mg. of N in 10 cc. for the solubility of the casein molecule.

NaOH added to 1 gm. casein.	Protein N in 100 cc.			Protein N in 100 cc.		
	80 *mg.*	160 *mg.*	320 *mg.*	80 *mg.*	160 *mg.*	320 *mg.*
	Protein N dissolved in 10 cc.			Protein N dissolved by 1×10^{-5} mols NaOH.		
mols $\times 10^{-5}$	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
1.0	0.29	0.43	0.75	3.20	3.00	3.10
2.0	0.43	0.73	1.34	3.00	3.00	3.02
3.0	0.57	0.98	1.89	2.93	2.83	2.93
4.0	0.74			3.05		
5.0	0.91	1.73		3.12	3.20	
8.0	1.35	2.95	5.98	3.05	3.52	3.66
10.0	1.77	3.37	6.50	3.28	3.24	3.19
15.0	2.51	5.22	10.20	3.17	3.39	3.36
20.0	3.35	6.71	13.25	3.22	3.29	3.28

decreases with increase in total solubility, and a variable correction should therefore be applied.

The most probable equivalent combining ratio of casein for base is the average of the determinations when 10, 15, and 20×10^{-5} mols of sodium hydroxide were added to each gram of casein. These give 3.38 mg. of casein nitrogen to 1×10^{-5} mols of sodium hydroxide if no correction is applied. If 0.13 mg. of nitrogen in 10 cc. is allowed for the solubility of the casein molecule, 3.27 mg. of nitrogen combined with 1×10^{-5} mols of base. If each gram of casein contains 0.156 mg. of nitrogen (27), each mol of sodium hydroxide combined with less than 2,166 gm. and

probably with 2,096 gm. of casein. In round numbers, 2,100 may therefore be taken as the combining weight.

This is in close accord with the result of Yamakami (14) determined in a somewhat similar manner. It is, however, approximately half the value determined by Robertson (6) and by Van Slyke and Bosworth (10). These investigators, however, determined the minimal base-binding capacity of casein in quite another way. They added hydrochloric acid to a solution of sodium caseinate until precipitation took place, and concluded that at the highest acid concentration that they could reach before casein precipitated the "base was saturated with protein." Yamakami has criticized this procedure and pointed out the globulin-like effect of the salt that accumulates in increasing the solubility of casein. Van Slyke and Bosworth attempted to dialyze during the precipitation, and believed that a monosodium compound of casein was stable under these conditions. The fact that the base bound under these circumstances was just half that bound in these experiments would seem to favor their contention. The equilibria involved in titrating casein with sodium hydroxide are, moreover, quite different from those involved in titrating sodium caseinate with hydrochloric acid; these will be considered at another time.

Besides these investigators a host of earlier workers have titrated casein to a hydrogen ion concentration neutral to litmus or to phenolphthalein. Reference may be made to Raudnitz (28) for a summary of their results. Their chief interest would lie in the very slight variation in the many casein preparations made from many different kinds of milk, were it not that it so happens that sodium hydroxide just carries casein into solution at a reaction neutral to litmus; and that again as much sodium hydroxide is required to bring the reaction to phenolphthalein. Because of this coincidence, and because of the nature of the equilibria that obtain between sodium hydroxide and the polyvalent casein molecule, the results of these titrations have approximated either simple multiples or denominators of the equivalent combining capacity of casein for base.

IX.

The Minimal Molecular Weight of Casein.

Laqueur and Sackur (2), Long (7), Robertson (6), Van Slyke and Bosworth (10), and Yamakami[14] (14) have all attempted to evaluate the minimal molecular weight of casein. For the most part, their calculations have depended upon the phosphorus and the sulfur content. Hammarsten (29) early determined the proportion of both of these elements in casein, and this value for sulfur has never been appreciably changed. Laqueur and Sackur found a lower content of phosphorus, and Bosworth and Van Slyke (30) a still lower one in a casein preparation that they believed had been adequately purified of calcium phosphate. The results of these elementary analyses are tabulated below (Table VIII), and from them has been calculated the

TABLE VIII.

Minimal Molecular Weight of Casein Calculated from Its Phosphorus and Sulfur Content.

	Hammarsten (29).	Laqueur and Sackur (2).	Bosworth and Van Slyke (30).	Weight of casein containing 1 gram atom.
	per cent in casein.			*gm.*
Phosphorus.	0.85	0.77	0.71	4,372
Sulfur.	0.76	0.76		4,218

smallest molecular weight that can be ascribed to casein in order that it may contain 1 gram atom each of phosphorus and sulfur.

The phosphorus and sulfur content of casein leads to a minimal molecular weight of approximately 4,300. This is twice the equivalent combining weight for sodium hydroxide that we have found. Even in the neighborhood of its isoelectric point casein must therefore behave as a divalent acid. Laqueur and Sackur (2), Long (7), and Robertson (6) suspected that a still greater number of acid groups were involved at a reaction neutral to litmus or to phenolphthalein from conductivity data.

[14] Yamakami also attempted to determine the molecular weight of casein by Barger's method. He believed the molecular weight to be about 4,000 and the equivalent weight about 2,000.

The proportions of amino-acids that casein yields upon hydrolysis indicate that its molecular weight must be far greater than 4,300. Osborne and Guest (27) made a critical study of the available data relating to the amino-acids found in casein in 1911. Since then

TABLE IX.

Minimal Molecular Weight of Casein Calculated from Its Amino-Acid Content.

Amino-acid.	Osborne and Guest (27).	Van Slyke (31).	Dakin* (32).	Foreman (33).	Folin and Looney (34).	Assumed value.	Weight of casein containing 1 gram molecule.	No. of assumed molecules.	Calculated minimal molecular weight.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*			
Glycocoll.	0.00			0.45					
Alanine.	1.50			1.85		1.85	4,814	3	14,442
Valine.	7.20			7.93		7.93	1,477	9	13,293
Leucine.	9.35			9.7		9.7	1,352	10	13,520
Proline.	6.70		8.7	7.63		8.7	1,323	10	13,230
Phenylalanine.	3.20			3.88		3.88	4,255	3	12,765
Glutamic acid.	15.55		21.6	21.77		21.77	675	19	12,825
Aspartic "	1.39		4.1	1.77		4.1	3,246	4	12,984
Lysine.	5.95	8.38				8.38	1,743	7	12,201
Serine.	0.50		Trace.						
Tyrosine.	4.50				5.3	5.3	3,417	4	13,668
Hydroxyproline.	0.23								
Cystine.					0.25	0.25	96,000		
Histidine.	2.50	2.53				2.50	6,204	2	12,408
Arginine.	3.81	3.59				3.81	4,520	3	13,710
Tryptophane.	1.50		1.7		1.54	1.5 1.7	13,600 12,000	1 1	13,600 12,000
β-Hydroxyglutamic acid.			10.5			10.5	1,550	8	12,400
Ammonia.	1.61					1.61	1,060	12†	12,720

* Where several values are given representing the yields in different preparations the highest has generally been taken.

† It is significant to note that the number of ammonia molecules falls far short of the number necessary to form amides of the disbasic acids. Dr. Dakin suggests that it is therefore a fair inference that a large proportion of the glutamic and β-hydroxyglutamic acids exists as cyclic anhydrides in the casein molecule. See also Vickery (35).

D. D. Van Slyke has redetermined the hexone bases (31); Dakin (32), and Foreman (33) have increased the yields of several of the acids by improved methods of hydrolysis and separation; and Folin and Looney (34) have determined cystine, tyrosine, and tryptophane by new colorimetric methods. The results of these different investigations are collected in the appended table (Table IX).

The tryptophane content of casein is now probably extremely well known. If it is present to the extent of 1.7 per cent (Dakin),[15] the molecular weight of casein must be at least 12,000, according to the following method of calculation

$$\frac{\text{Weight of amino-acid found}}{\text{Molecular weight of amino-acid}} = \frac{\text{Weight of casein taken}}{\text{Molecular weight of casein}}$$

If casein contains only 1.5 per cent of tryptophane, however, the molecular weight must be 13,600. Three times the minimal molecular weight calculated from the phosphorus and sulfur, respectively, leads to a molecular weight of 13,320 and 12,630. Six times the equivalent combining weight of casein for base leads to the value 12,600. Each of these calculations involves an independent set of determinations. Their average may therefore be taken as the tentative minimal molecular weight of casein.

TABLE X.

Minimal molecular weight of casein calculated from tryptophane (Dakin)	12,000
" " " " " " " " (Folin and Looney)	13,600
Minimal molecular weight of casein calculated from tryptophane (average)	12,800
" " " " " " " phosphorus × 3	13,116
" " " " " " " sulfur × 3	12,654
Equivalent combining weight of casein for sodium hydroxide × 6	12,600
Average	12,792

It is true that casein may have a still higher molecular weight. Serine, oxyproline, and cystine have all been isolated from, or determined in, the protein molecule in such small amounts that a much larger molecular weight would have to be assumed in order

[15] Fürth and Nobel (36), and more recently Komm and Boehringer (37), believe that casein contains a still higher percentage of tryptophane. The excellent agreement between the different determinations here recorded, and also those of Hopkins and Cole (38), Thomas (39), and May and Rose (40), demands that the lower values be retained until further evidence is presented.

that casein might contain 1 molecule of each of them. The value of cystine depends not upon isolation, but upon the new colorimetric determination of Folin and Looney (34). If it is correct, the molecular weight of casein cannot be less than 96,000. The early study of T. B. Osborne (41) of the distribution between the different kinds of sulfur in the casein molecule also suggested a somewhat higher molecular weight. For the present, however, it seems preferable to await further investigations before raising the molecular weight of casein to more than six times the equivalent weight.

The number of molecules of the other amino-acids that would exist in casein if its molecular weight were 12,800 is calculated in the last columns of Table IX. The number of cases where the product of the weight of casein that could contain 1 molecule of the amino-acid, by an assumed small number of molecules, leads to a weight near this, must be considered as additional evidence in favor of this value, or some multiple of it.

X.

The Dissociation of Casein.

The casein that we have used was prepared by causing it to flock at that hydrogen ion concentration which has generally been considered the isoelectric point of casein. Michaelis and Pechstein (42) give this hydrogen ion concentration as 2.4×10^{-5} N, and our own experience confirms their judgment that this reaction is the most favorable one for the precipitation of casein.

In the first of these studies we pointed out that the hydrogen ion concentration produced in water by the dissociation of a pure protein was often somewhat lower than that produced by the dissociation of its compounds.[16] Our measurements indicate that the hydrogen ion concentration of pure casein lies near 1.3×10^{-5} N. This difference in the hydrogen ion concentration of freshly precipitated and of purified casein might be explained in one of three ways. One might assume that the soluble compounds, the neutral salt, and the slight excess of acid, that were present when casein was precipitated, had depressed the unequal dissociation of this protein as an acid and as a

[16] Cohn (17), p. 705.

base, and that their removal therefore brought about the slight decrease in hydrogen ion concentration. Or one might assume that the point of flocculation represented the true isoelectric point, and that the change in reaction was brought about by the combination of casein as an acid with water. Finally, one might contend that a pure protein need not exist at its isoelectric point. This must be admitted if certain definitions of the isoelectric point are accepted.[17] It is for this reason that we have characterized our proteins when they were uncombined with acids and bases, when they dissolved in water to a constant and characteristic extent, and, as we have seen, produced a hydrogen ion concentration in water as a result of their dissociation.

If the solubility product constant, which we have evaluated from the solubility of casein in systems containing small amounts of sodium hydroxide, be divided by the square of the hydrogen ion concentration produced in water by uncombined casein, the quotient should represent that part of the solubility of the pure protein in water that was due to the divalent casein anion.

$$\frac{Su \cdot Ka_1 \cdot Ka_2}{(H^+)^2} = \frac{340 \times 10^{-12} \text{ mg. N in 1 liter}}{(1.3 \times 10^{-6})^2} = 2 \text{ mg. N in 1 liter at 25°C.}$$

This quantity was related to the observed solubility and to the solubility of the casein molecule in equation (6).

$$S = Su + \frac{Su \cdot Ka_1 \cdot Ka_2}{(H^+)^2} \qquad (6)$$

---

[17] Michaelis has characterized the isoelectric point of an ampholyte as the point (*a*) at which its dissociation is at a minimum, (*b*) its anion concentration is equal to its cation concentration, and (*c*) on one side of which it dissociates as an acid, on the other as a base.[18] The last definition is clearly applicable to the isoelectric point of casein. It is upon the first two, however, that the equations for dissociation near the isoelectric point depend. These have been applied to the proteins, often without reference to the simplifying assumptions employed in their deduction. The first two definitions are intimately related to each other. Michaelis showed that the dissociation of a simple ampholyte was at a minimum when the anion concentration was equal to the cation concentration. This important relation holds rigorously in the case of substances whose equivalent combining weight is identical for acids and for bases. The equivalent combining weight of casein for base is, however, greater than its combining weight for acid. As a result the minimum degree of dissociation probably occurs in systems containing a very small amount of acid.

[18] Michaelis (18), p. 39.

The second term, Su, represented the solubility of the casein molecule. Three different methods have yielded values for this portion of the solubility of casein that were in good agreement, and varied only from

$$Su = 13 \text{ to } 15 \text{ mg. N in 1 liter at } 25°C.$$

The solubility (S) of uncombined casein in water has been very accurately determined and has previously been reported (17). The result of many analyses yielded the result

$$S = 17.8 \pm 0.6 \text{ mg. N in 1 liter at } 25°C.$$

In deducing an equation that would characterize and define the solubility of casein in systems containing base, we considered the solubility due to the basic dissociation of casein negligible. The results that we have obtained have justified this simplifying assumption. The assumption can, however, not be expected to hold in the case that we are now considering; namely, the solubility of casein in water. The solubility due to soluble casein cations is therefore indicated by the difference between the observed solubility of casein in water and the sum of the calculated solubilities of the casein molecule and the divalent casein anion. This quantity cannot, it is true, be very exactly estimated from these data. If the highest value for the total solubility and for the solubility of the casein molecule is considered it becomes

$$S - Su - \frac{Su \cdot Ka_1 \cdot Ka_2}{(H^+)^2} = 18.4 - 15 - 2 = 1.4 \text{ mg. N in 1 liter at } 25°C.$$

while consideration of the lower value for S and for Su leads to the conclusion that as much of the solubility of casein in water is due to basic as to acid ions

$$S - Su - \frac{Su \cdot Ka_1 \cdot Ka_2}{(H^+)^2} = 17.2 - 13 - 2 = 2.2 \text{ mg. N in 1 liter at } 25°C.$$

The present data are not sufficiently accurate to allow us to formulate any conclusion other than that both of these soluble ions exist, and are present almost to the same extent.

Finally, we can estimate the strength of the dissociation of casein as an acid in terms of the product of the dissociation constants Ka_1

and Ka_2. This product results, if we substitute our value for the solubility of the casein molecule, in the solubility product constant. The higher and lower values for the solubilty of the casein molecule indicate that the product of these constants must lie between 24 and 26×10^{-12} N. Even if these constants were only of the same strength—and the one is sufficiently great to enable casein to produce a hydrogen ion concentration in water of 1.3×10^{-5} N—our results indicate that casein is a relatively strong acid. Strong enough, as early investigators showed, to have "the power of expelling carbonic anhydride from soluble carbonates and bicarbonates."[3]

XI.

SUMMARY.

1. The solubility in water of purified, uncombined casein has previously been reported to be 0.11 gm. in 1 liter at 25°C. This solubility represents the sum of the concentrations of the casein molecule and of the soluble ions into which it dissociates.

2. The solubility of casein has now been studied in systems containing the protein and varying amounts of sodium hydroxide. It was found that casein forms a well defined soluble disodium compound, and that solubility was completely determined by (*a*) the solubility of the casein molecule, and (*b*) the concentration of the disodium casein compound.

3. In our experiments each mol of sodium hydroxide combined with approximately 2,100 gm. of casein.

4. The equivalent combining weight of casein for this base is just half the minimal molecular weight as calculated from the sulfur and phosphorus content, and one-sixth the minimal molecular weight calculated from the tryptophane content of casein.

5. From the study of systems containing the protein and very small amounts of sodium hydroxide it was possible to determine the solubility of the casein molecule, and also the degree to which it dissociated as a divalent acid and combined with base.

6. Solubility in such systems increased in direct proportion to the amount of sodium hydroxide they contained.

7. The concentration of the soluble casein compound varied inversely as the square of the hydrogen ion concentration, directly as the solubility of the casein molecule, Su, and as the constants Ka_1 and Ka_2 defining its acid dissociation.

8. The product of the solubility of the casein molecule and its acid dissociation constants yields the solubility product constant, $Su \cdot Ka_1 \cdot Ka_2 = 2.2 \times 10^{-12}$ gm. casein per liter at 25°C.

9. The solubility of the casein molecule has been estimated from this constant, and also from the relation between the solubility of the casein and the sodium hydroxide concentration, to be approximately 0.09 gm. per liter at 25°C.

10. The product of the acid dissociation constants, Ka_1 and Ka_2, must therefore be 24×10^{-12}N.

11. It is believed that these constants completely characterize the solubility of casein in systems containing the protein and small amounts of sodium hydroxide.

BIBLIOGRAPHY.

1. Cohn, E. J., *J. Biol. Chem.*, 1922, l, p. ix.
2. Laqueur, E., and Sackur, O., *Beitr. chem. Physiol. u. Path.*, 1903, iii, 193.
3. Osborne, W. A., *J. Physiol.*, 1901–02, xxvii, 398.
4. Robertson, T. B., *J. Biol. Chem.*, 1906–07, ii, 317.
5. Robertson, T. B., *J. Phys. Chem.*, 1911, xv, 179.
6. Robertson, T. B., The physical chemistry of the proteins, New York, London, Bombay, Calcutta, and Madras, 1918.
7. Long, J. H., *J. Am. Chem. Soc.*, 1906, xxviii, 372.
8. Van Slyke, L. L., and Hart, E. B., *Am. Chem. J.*, 1905, xxxiii, 461.
9. Bosworth, A. W., and Van Slyke, L. L., *J. Biol. Chem.*, 1913, xiv, 207.
10. Van Slyke, L. L., and Bosworth, A. W., *J. Biol. Chem.*, 1913, xiv, 211.
11. Van Slyke, L. L., and Bosworth, A. W., *J. Biol. Chem.*, 1913, xiv, 227.
12. Van Slyke, L. L., and Winter, O. B., *J. Biol. Chem.*, 1914, xvii, 287.
13. Bosworth, A. W., and Van Slyke, L. L., *J. Biol. Chem.*, 1916, xxiv, 173.
14. Yamakami, K., *Biochem. J.*, 1920, xiv, 522.
15. Clark, W. M., *J. Ind. and Eng. Chem.*, 1920, xii, 1162.
16. Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 547.
17. Cohn, E. J., *J. Gen. Physiol.*, 1921–22, iv, 697.
18. Michaelis, L., Die Wasserstoffionenkonzentration. Ihre Bedeutung für die Biologie und die Methoden ihrer Messung, Berlin, 1914.
19. Cohn, E. J., *J. Biol. Chem.*, 1921, xlvi, p. iii.
20. Van Slyke, L. L., and Baker, J. C., *J. Biol. Chem.*, 1918, xxxv, 127.

21. Sörensen, S. P. L., *Compt. rend. trav. Lab. Carlsberg*, 1917, xii.
22. Henderson, L. J., and Cohn, E. J., *J. Am. Chem. Soc.*, 1918, xl, 857.
23. Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 1922.
24. Bruner and Zawadzki, *Bull. Acad. Sc. Cracovie*, July, 1909, quoted from Lewis (25), I, p. 305.
25. Lewis, W. C. McC., A system of physical chemistry, New York, London, Bombay, Calcutta, and Madras, 1st edition, 1916, I, II.
26. Kraus, C. A., The properties of electrically conducting systems, New York, 1922.
27. Osborne, T. B., and Guest, H. H., *J. Biol. Chem.*, 1911, ix, 333.
28. Raudnitz, R. W., *Ergebn. Physiol.*, 1903, ii, *Ite Abt.*, 193.
29. Hammarsten, O., *Z. physiol. Chem.*, 1882–83, vii, 227.
30. Bosworth, A. W., and Van Slyke, L. L., *J. Biol. Chem.*, 1914, xix, 67.
31. Van Slyke, D. D., *J. Biol. Chem.*, 1913–14, xvi, 531.
32. Dakin, H. D., *Biochem. J.*, 1918, xii, 290.
33. Foreman, F. W., *Biochem. J.*, 1919, xiii, 378.
34. Folin, O., and Looney, J. M., *J. Biol. Chem.*, 1922, li, 421.
35. Vickery, H. B., *J. Biol. Chem.*, 1922, liii, 495.
36. Fürth, O., and Nobel, E., *Biochem. Z.*, 1920, cix, 103.
37. Komm, E., and Boehringer, E., *Z. physiol. Chem.*, 1923, cxxiv, 287.
38. Hopkins, F. G., and Cole, S. W., *J. Physiol.*, 1901–02, xxvii, 418.
39. Thomas, P., *Ann. Inst. Pasteur*, 1920, xxxiv, 701.
40. May, C. E., and Rose, E. R., *J. Biol. Chem.*, 1922, liv, 213.
41. Osborne, T. B., *Z. anal. Chem.*, 1902, xli, 25.
42. Michaelis, L., and Pechstein, H., *Biochem. Z.*, 1912, xlvii, 260.

SENSORY ADAPTATION AND THE STATIONARY STATE.

By SELIG HECHT.*

(From the Department of Physical Chemistry in the Laboratories of Physiology, Harvard Medical School, Boston.)

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I.

The purpose of the present investigation is to determine the nature of the changes in sensitivity which take place in a light sensitive animal when it is exposed to different conditions of illumination. Although the superficial changes which animals undergo under these conditions are known in a general way, the inner changes which make possible these outer, more obvious differences of sensitivity, are not known at all. In order to discover these basic, inner changes, it is necessary to know the nature of the physicochemical system which composes the sensory mechanism. Since the essential kinetics and dynamics of such a system have, as an hypothesis at least, been described for the light sensitivity of the clam, *Mya arenaria*, it seems logical, and indeed necessary, to determine the behavior of the system under the commonest conditions of its existence; namely, its adaptation to different intensities of illumination.

One phase of this problem has already been studied—the adaptation to complete absence of light (Hecht, 1918–19, *a*). As a result, a certain mechanism was postulated in explanation of the changes found experimentally. The actual method of analysis of the present experiments will, therefore, be to determine whether this mechanism, originally suggested for the special instance of the process of adaptation to zero intensity, may be carried over to the more general situation of the adaptation to light of any intensity, and in particular to decide, if possible, between certain alternatives presented in the explanation of dark adaptation alone.

* National Research Fellow.

In order to do this it will be necessary to treat the data mathematically with a degree of detail that is possible only because the experiments with *Mya* give results that are dependable quantitatively. It has seemed advisable to do this because the photosensory activity of the clam is in no fundamental way different from the sensitivity of other animals to light, and indeed to other forms of stimulation. Certainly the ability to become adapted to sustained stimulation is characteristic of all sense organs. So that while the minute analysis of one sensory response does not presuppose an identical mechanism in another, the basic ideas derived are of general interest. I have therefore pushed the analysis of the present experiments to their full capacity because of the belief that the only general value and perhaps the main justification of a series of experiments lie in their complete theoretical treatment in terms of certain fundamental concepts.

II.

1. *Mya*, when exposed to light, indicates its stimulation by retracting its siphons after a definite time. If the illumination is maintained no further response takes place, and the animal becomes adapted. Its sensitivity has, however, become changed, in that a longer exposure than before is necessary to elicit a response to an intensity higher than the one to which it is adapted. The experiments have consisted essentially in allowing an animal to become adapted to each of a series of intensities between 0 and 530 meter candles, and in measuring the exposure necessary to produce a response at each of the adaptation intensities.

The actual procedure is of the following nature. A thoroughly dark adapted animal is placed at a given distance from the adapting light, to which it remains exposed undisturbed for 15 minutes. It is then illuminated by the stimulating light and its reaction time noted. 6 minutes are allowed to elapse, during which the animal continues to remain exposed to the adapting light alone, and at the end of which the reaction time is again measured to the stimulating light. A 6 minute period is again allowed, and a third reaction time measurement is made. The animal is then placed in the dark for at least half an hour, after which it is adapted to a different intensity, and

three readings are made of its reaction time to the same stimulating light as before. It is again given a half hour stay in the dark, and adapted to still another intensity. A single experiment consists in carrying an animal through a series of seven or eight adapting intensities and securing triplicate measurements of its reaction time to the same stimulating light. The order in which the adapting intensities are tested is purposely haphazard.

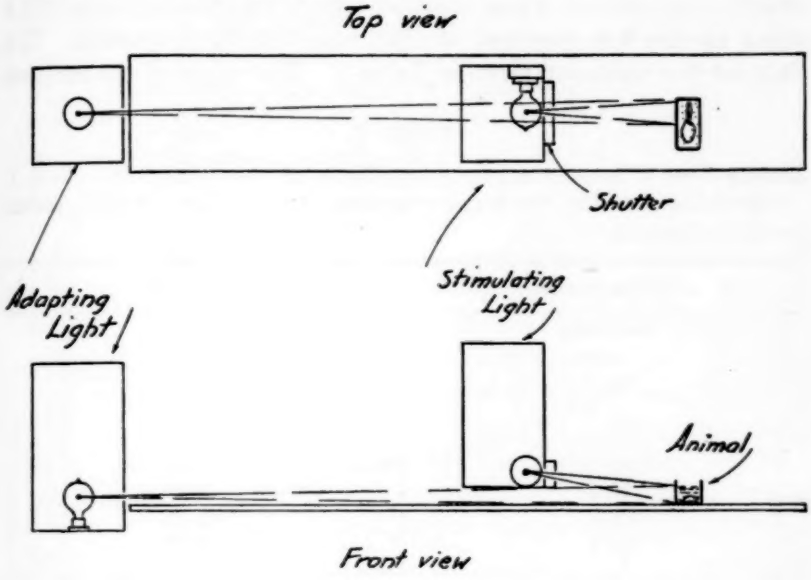

FIG. 1. Diagrammatic representation of the apparatus for use in adaptation and stimulation experiments.

The range of intensities is secured by using as different sources of illumination three concentrated filament lamps—a 15 watt, a 250 watt, and a 400 watt Mazda—whose candle powers were calibrated photometrically. The animals are placed at such distances from the lamps as to give the proper illumination, calculated on the inverse square law. The stimulating light is also a 250 watt concentrated filament lamp, and is at a constant distance from the animal regardless of the latter's distance from the adapting light. The stimulating light is placed just above the illuminating beam from the adapting

source so that it does not interfere with the adapting light. This arrangement is illustrated diagrammatically in Fig. 1. The exposure of the animal to the stimulating light is controlled by a shutter, and the reaction time is measured with a stop-watch. The temperature is kept constant by changing the sea water during the rest period in the dark.

2. I performed two series of experiments.[1] In the first I used twenty-three animals whose reaction time to an illumination of 694.4 meter candles was measured at eight intensities of adaptation. The data for this series are given in Table I. The values of the reaction

TABLE I.

Reaction Time to Stimulation after Adaptation to Different Intensities. Series I. Stimulating intensity, 694.4 meter candles. Temperature, 19.7°C. Latent period, 1.27 seconds.

Adapting intensity.	Reaction time.
meter candles	*sec.*
0.00	1.67
0.976	1.93
2.57	1.97
8.78	2.01
27.78	2.14
73.04	2.24
250.0	2.39
530.6	2.78

time are the averages of 69 measurements, 3 with each of twenty-three animals. After this series was completed, and the general appearance of the data ascertained, I performed the second series with sixteen animals, covering the same range of intensities as the first, but in seven steps instead of eight. However, the stimulating light was much more intense—2,778 meter candles. The results are given in Table II, where the values of the reaction time are the averages of 48 measurements, 3 with each of sixteen animals.

[1] These experiments were made at the Marine Biological Laboratory at Woods Hole during the summer of 1922. I wish to express my thanks to the Director, Dr. Frank R. Lillie, for placing laboratory space at my disposal.

TABLE II.

Reaction Time to Stimulation after Adaptation to Different Intensities. Series 2. Stimulating intensity, 2,778 meter candles. Temperature, 20.6°C. Latent period, 1.10 seconds.

Adapting intensity.	Reaction time.
meter candles	*sec.*
0.00	1.44
0.976	1.56
5.20	1.68
27.78	1.75
69.25	1.82
250.0	1.96
530.6	2.22

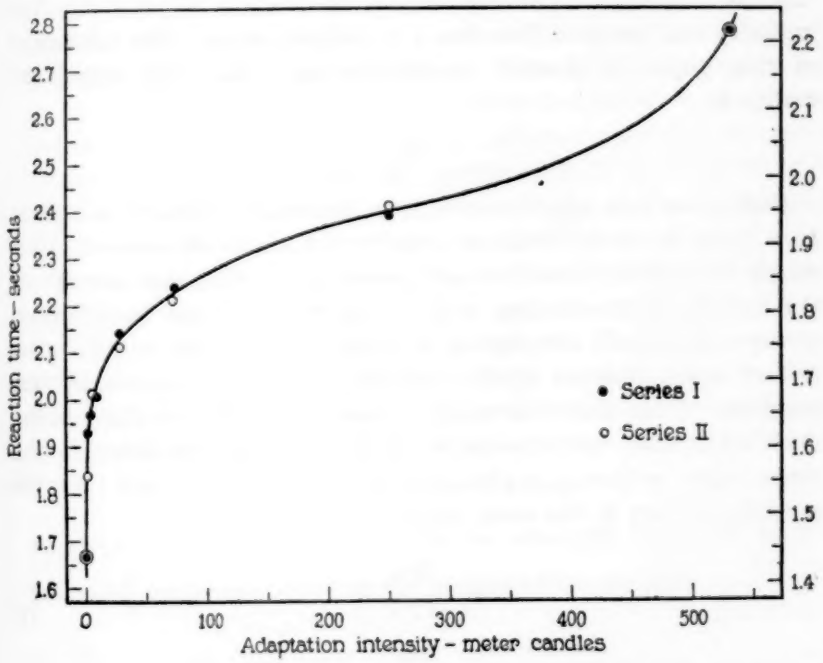

FIG. 2. Results of adaptation experiments. The scales of the ordinates are arranged so that both series of results take up the same vertical distance. The two series of experiments evidently give the same results.

The purpose of the second series of experiments was to see whether the results obtained in the first set were real or only apparent. The data of the first series are plotted in Fig. 2, and it must be apparent that the relationship between the intensity of adaptation and the reaction time is a curious one. The curve shows a point of inflection, it being first concave and then convex to the axis of abscissæ. Is this relationship an accidental one depending on a fortuitous combination of intensities of adaptation and stimulation, or does it represent something real? Fig. 2 shows unmistakably that we are dealing with a real phenomenon. The ordinates at the left are those for the first series, whereas the ordinates at the right are for the second series arranged to such a scale that the vertical distance between the two extremes of reaction time is the same for both series of experiments. It is clear that the results are the same in the two cases, and therefore that the shape of the curve expressing the relation between adapting intensity and reaction time has a real significance. The remainder of this paper is devoted to ascertaining what this significant reality is.

III.

During the dark adaptation of *Mya*, the reaction time of an animal to a given intensity decreases steadily for about 30 minutes, after which the reaction time becomes constant. Taking due account of the division of the reaction time into latent period and sensitization or exposure period, the relation between reaction time and duration of dark adaptation can be expressed by the isotherm of a bimolecular reaction. From this I concluded (Hecht, 1918–19, *a*) that during dark adaptation two substances, P and A, are combining in the sense organ to form the photosensitive substance, S, and that the sensitive system in the sense organ is

$$S \underset{\text{"dark"}}{\overset{\text{light}}{\rightleftharpoons}} P + A \qquad (1)$$

which represents a reversible photochemical reaction similar to the polymerization of anthracene (Luther and Weigert, 1905).

There are two ways in which such a reversible reaction can be derived from the data of dark adaptation. The first assumes (*a*) that the photochemical decomposition of S is directly proportional to the exposure, and (*b*) that in order to produce a response, the amount of freshly formed P and A must be a constant fraction of the amount of P and A already present in the sense organ. The exposure thus represents the actual amount of photochemical decomposition. Since the ratio between fresh and residual P and A is constant, the changes in reaction time measure the changes in concentration of residual P and A. Because the former follows a bimolecular isotherm, the latter must do so also, and the formation of sensitive substance, S, from its precursor, P, and its accessory, A, is $P + A \rightarrow S$.

The second interpretation of the data assumes (*a*) that the velocity of decomposition of S is directly proportional to its concentration, and (*b*) that in order to produce a response a constant quantity of fresh P and A must be formed in the sense organ. Accordingly, the variations in the exposure represent differences in the velocity of formation of this constant amount. The velocity is proportional to the reciprocal of the exposure, and since this follows a bimolecular isotherm, the velocity also changes according to a bimolecular curve. Because the velocity is directly proportional to the concentration of S, the concentration of S increases according to the kinetics of a bimolecular process, and we have again that $P + A \rightarrow S$.

The critical point in the two interpretations depends upon whether we use the concentration of P and A proportional to the exposure directly, or whether we use the concentration of S as proportional to the reciprocal of the exposure. Both, however, follow a bimolecular curve. This is mathematically possible because the bimolecular isotherm is a hyperbola. The general equation of a hyperbola is

$$Ax + By + Cxy + D = 0 \tag{2}$$

in which x, let us say, represents the exposure time. If we take instead $\frac{1}{x}$ or the reciprocal of the exposure time, we get

$$\frac{A}{x} + By + \frac{Cy}{x} + D = 0. \tag{3}$$

which on multiplying through becomes

$$Dx + Cy + Bxy + A = 0. \tag{4}$$

This obviously is also an hyperbola; its axes are slightly different from those of equation (2), but it is readily transformed into the equation of a bimolecular reaction.[2]

Given the fact that the relation between reaction time (properly corrected for latent period) and time of adaptation is bimolecular, shall we assume that the necessary exposure measures the photochemical effect directly, or that it measures the velocity of the photochemical reaction? The former gives the constant ratio; the latter gives the constant quantity; and on the basis of the data of dark adaptation alone, a choice between the two is not possible. Moreover, in all the other investigations with *Mya* it makes no difference which idea is valid, because the results are identical. The experiments are always with completely dark adapted animals. The amount of P and A in the sense cells is therefore minimal and constant, and a constant fraction of a constant minimum yields a constant quantity. The fact that for short exposures near the beginning of the reaction $S \rightarrow P + A$ there is a linear relation between exposure and effect (Hecht, 1918–19, *b;* 1920–21, *d*) proves neither assumption, because almost any curve may be considered a straight line over small distances. I have therefore arranged the present set of experiments in the hope that they would enable us to decide between the alternative explanations.

[2] It is curiously significant that these two interpretations are possible only because the equation relating reaction time and adaptation time is of the second order. At the beginning of the work with *Mya*, the constant ratio idea interested me because of its possible bearing on the Weber-Fechner relation, and I adopted it without further comment not only for *Mya* but for the human eye as well (Hecht, 1921–22, *e*). I have since become convinced, however, that Weber's law will find its explanation quite differently, as the present paper undoubtedly shows. It is therefore no longer any reason for making a choice between the two ideas, and I have been led to reconsider the constant quantity idea to the extent of making the preliminary experiments for the present paper in the summer of 1920. In addition, Dr. E. Q. Adams of the Nela Research Laboratories has recently urged upon me the advisability of adopting the constant quantity idea in connection with my experiments on the dark adaptation of the fovea of the human eye, and has in fact made the necessary computations for it. I take this opportunity for thanking Dr. Adams for his interest in this matter and for his kindness in communicating his ideas to me.

IV.

1. No matter which of the two working arrangements we adopt for it, the basis of the photosensory system in *Mya* is still the reversible photochemical reaction

$$S \underset{\text{"dark"}}{\overset{\text{light}}{\rightleftharpoons}} P + A \qquad (1)$$

From our general knowledge of the photochemistry of such reactions we can determine what will take place in this system under different conditions of illumination. When the system is exposed to a constant illumination, such as will produce adaptation in the animal, the light is absorbed by the sensitive material, S, which is then decomposed to form P and A. Since the reverse reaction is independent of light, it will proceed according to the ordinary mass law. As soon as some P and A are formed, they will unite to form S again, and the velocity of formation will depend on the concentration of P and A. The sensitive material S will continue to be broken down by the light, and the precursors P and A will continue to form S as long as the illumination is maintained, until a stationary state is reached in which the rate of photolysis of S is balanced by the regenerative "dark" formation of S from P and A. In this stationary condition the concentrations of S, P, and A will remain constant as long as the light continues to shine on the animal.

For purposes of calculation it is necessary to describe these processes more rigorously. By doing so it is possible to test the two interpretations previously suggested, in order to determine which is consistent with the present experimental findings. I shall consider the "constant ratio" idea first, because, though the mathematical treatment is practically the same in both cases, the equations involved are somewhat simpler than those for the "constant quantity" idea. It will be recalled that the crucial assumption of the constant ratio idea is that the velocity of decomposition of S is constant. Since the effect of light is proportional to its absorption, this assumption means that the concentration of S remains sensibly constant in comparison to that of its decomposition products.

The reaction system has been illuminated for the time, t, during which x units of S have been decomposed to form an equivalent amount of P and A. The velocity of the "light" reaction $S \rightarrow P + A$ is

$$\frac{\partial x}{\partial t} = k_1 \tag{5}$$

where k_1 is the velocity constant. The reverse "dark" reaction $S \leftarrow P + A$, being independent of light follows the mass law, and its velocity is

$$-\frac{\partial x}{\partial t} = k_2 x^2 \tag{6}$$

in which k_2 is the velocity constant, and the sign is negative because the velocity is opposite in direction to that of equation (5). The sum of these two partial velocities

$$\frac{dx}{dt} = k_1 - k_2 x^2 \tag{7}$$

gives the real velocity of formation of P and A from S when both reactions are going on.

When the reaction $S \rightleftharpoons P + A$ has reached a stationary state, which represents the condition when the animal has become adapted, there is no further change in the concentrations of S, P, and A, and the velocity $\frac{dx}{dt} = 0$. Equation (7) then becomes

$$k_1 - k_2 x^2 = 0 \tag{8}$$

which on rearrangement of terms is

$$x = \sqrt{\frac{k_1}{k_2}} \tag{9}$$

In equation (9) k_2 is constant under all conditions of illumination since it is the velocity constant of the "dark" reaction. The value of k_1, though constant for a given illumination, varies with the intensity I. The simplest relation between I and k_1 is a linear one. Therefore let us write

$$k_1 = m I \tag{10}$$

where m is a constant of proportionality. Substituting this value of k_1 in equation (9) we get

$$x = \sqrt{\frac{m\,I}{k_2}} \tag{11}$$

which describes completely the concentration x of P and A at the stationary state induced by any intensity, I, to which the animal is adapted.

To produce a response in the animal after it has thus become adapted, it must be exposed to the stimulating light. The sensory system is then illuminated by an intensity equal to the sum of the stimulating and adapting intensities. Due to this exposure the stationary state is upset, and fresh P and A are formed by the decomposition of S. The velocity of formation of P and A is given by equation (7), in which, however, k_1 has now a value corresponding to the increased intensity of illumination as given by equation (10). In order to determine the amount of P and A formed during the course of this reaction it is necessary to integrate equation (7). The integrated form is

$$t = \frac{1}{\sqrt{k_1 k_2}} \tanh^{-1}\left(x\sqrt{\frac{k_2}{k_1}}\right) \tag{12}$$

and in terms of it we can determine the concentration x of P and A for any time, t, after the beginning of the reaction.

2. The thing which we wish to know is how much freshly formed P and A is necessary to produce stimulation in the animal. To find this we adopt the following procedure. At the stationary state the concentration of P and A is given by x in equation (11). When the new reaction, proceeding according to equation (12), is initiated as a result of the added illumination, it will begin at that point in its course which corresponds to a concentration x of P and A. It will then go on for a period of time which we know experimentally, and at the end of which the requisite amount of freshly formed P and A will have accumulated in the sense organ. Knowing this exposure period (reaction time minus latent period) and knowing the value of x, we are able to calculate the amount of P and A formed by means of equation (12).

These relations are shown graphically in Fig. 3, in which the curve is the reaction according to equation (12). Let x_o be the concentration of P and A at the stationary state as found by equation (11), and let t_o be the time corresponding to this concentration x_o in equation (12). This then is the moment when the light is turned on.

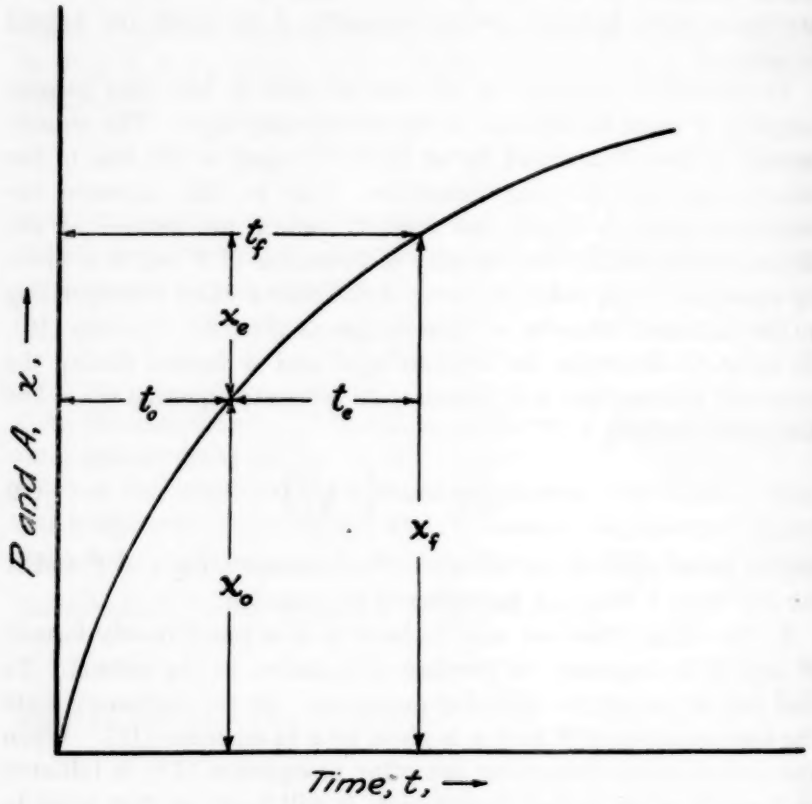

FIG. 3. Graphic representation of the mathematical procedure in determining the amount x_e of sensitive substance decomposed during the exposure period.

Let t_e be the exposure period, and t_f the sum of t_o and t_e. The reaction proceeds for the time t_e until the time t_f, at the end of which time an amount x_f of P and A will be present in the sense organ. To begin with, there were x_o units; at the end there are present x_f units. The difference between the two, $x_e = x_f - x_o$, will be the amount of P

and A freshly formed during the exposure t_e in order to produce a retraction reflex.

The initial concentration x_o of P and A at the stationary state depends on the adaptation intensity. The final concentration x_f depends not only on this initial concentration but on the stimulating light and on the exposure period. In order, however, for the present series of equations to be consistent, the amount x_e of the freshly accumulated P and A must be proportional to the amount x_o present at the stationary state. This is the "constant ratio" requirement. The calculation of the value of x_e from the experimental data is therefore critical in that it will prove or disprove the assumption on which the series of equations depends.

3. Inspection of equations (11) and (12) shows that they contain three variables—intensity I, time t, and concentration x. Given the numerical values of I and t, it is possible to solve the two equations for values of x_o, x_e, and x_f in terms of the constants m and k_2. This will determine whether x_e is proportional to x_o, as our assumption requires, or indeed, how in general x_e is related to x_o. The algebraic burden is lightened considerably because we have no interest in the absolute values of x_e. Numerical values for x_o and x_e are meaningless in themselves, since we know nothing of the actual materials involved and their concentrations. Therefore we can ascertain the way in which x_e varies with the adapting intensity and with x_o by assigning purely arbitrary numerical values to m and k_2. Changing the numerical magnitudes of m and k_2 is the equivalent of changing the units in which time and intensity are measured; and since all units are purely arbitrary, any values for m and k_2 will give the same answer to the question that concerns us.

Let us assume that the velocity constant of the bimolecular "dark" reaction $S \leftarrow P + A$ is $k_2 = 0.001$, and that $m = 0.01$—values that are obviously arbitrary. From equation (11) we then compute the concentration x_o of P and A present at the stationary state induced in the adaptation to any light intensity. The value of k_1 to be used in the corresponding equation (12) is derived from equation (10), remembering that the illumination of the animal during stimulation is the sum of the adapting and stimulating intensities. We thus secure the value of t_o representing the initial moment in the course of

the stimulating reaction. Adding to this value the exposure period t_e we get t_f. The exposure period is found by subtracting the latent period from the reaction time given in Tables I and II. For Series 1 the latent period is 1.27 seconds; for Series 2 the latent period is 1.10 seconds, the difference between the two being due to the difference in the temperature for the two series, as noted in Tables I and II (cf. Hecht, 1918–19, *c*). By means of the same equation (12)

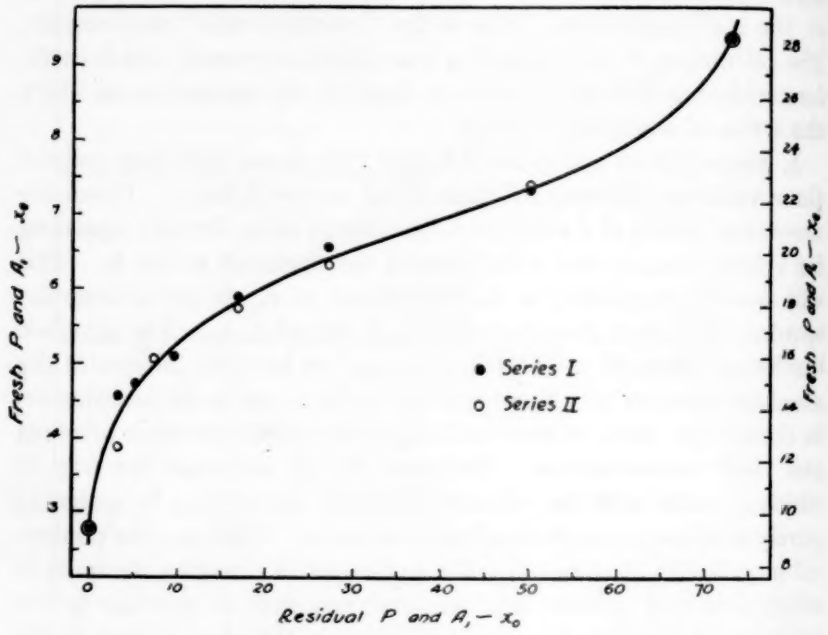

FIG. 4. Relation between residual P and A, and P and A freshly formed during the exposure. It is obvious that the relation between the two is not linear.

x_f is found from t_f. The difference between x_f, the final concentration, and x_o, the initial concentration, gives x_e, the concentration of freshly formed P and A necessary to produce stimulation.

According to the interpretation which we are testing in this section, x_e should be proportional to x_o. Fig. 4 gives in graphic form the values of x_o and x_e for both series of experiments. If the concentration of freshly formed P and A were directly proportional to the concentration of P and A already present, the points in Fig. 4 would all lie on

a straight line. It is apparent that this is true only for the middle portion of the curve, but that over the whole range of adapting intensities from 0 to 530 meter candles this relation breaks down. The relation between fresh and residual P and A follows in a general way the relation between intensity and reaction time as shown in Fig. 2. The analysis of the data in terms of the "constant ratio" idea therefore holds only for a small range of adaptation intensities, and is not consistent with the experiments over a large range of intensities.

It must be reiterated that the numerical values given in Fig. 4 are of no consequence in themselves. I have calculated the experiments using several sets of values for m and k_2, and in every instance the relation between x_o and x_e is the same as shown in Fig. 4. This is emphasized by the fact that the numerical magnitudes for Series 1 and 2 differ strikingly, yet the relation between residual and freshly formed P and A is the same, as the superposition of the points in Fig. 4 amply indicates. Therefore it must be concluded that the "constant ratio" idea is not capable of interpreting the entire experimental data consistently. It should therefore be abandoned, particularly if the alternative "constant quantity" idea is more successful in giving a reasonable account of the data.

V.

1. The critical assumption of the "constant quantity" idea is that in the system $S \rightleftarrows P + A$ the velocity of photochemical decomposition of S varies with its concentration. Let a be the initial concentration of S in the dark, when no P and A have been formed. Let the reaction proceed under the influence of light for the time t, during which x units of P and A have been formed. The velocity of the reaction $S \rightarrow P + A$ alone will then be

$$\frac{\partial x}{\partial t} = k_1 (a - x) \tag{13}$$

and the reverse reaction $S \leftarrow P + A$ will be

$$-\frac{\partial x}{\partial t} = k_2 x^2 \tag{14}$$

as before. Combining the two, we get

$$\frac{dx}{dt} = k_1 (a - x) - k_2 x^2 \tag{15}$$

as representing the actual velocity when both reactions go on. For the stationary state when $\frac{dx}{dt} = 0$ equation (15) becomes

$$\frac{k_1}{k_2} = \frac{x^2}{a - x} \tag{16}$$

Remembering that $k_1 = m\,I$ as previously, we get

$$\frac{m\,I}{k_2} = \frac{x^2}{a - x} \tag{17}$$

which describes the concentrations of S, P, and A during the stationary state produced by the adaptation of *Mya* to any intensity. When the animal is illuminated by the stimulating light, fresh P and A will be formed. The velocity of formation is given by equation (15), in which k_1 has a new value corresponding to the sum of the adapting and stimulating intensities. When integrated, equation (15) becomes

$$t = \frac{2}{\sqrt{q}} \tanh^{-1}\left(\frac{2\,k_2 x + k_1}{\sqrt{q}}\right) - \frac{2}{\sqrt{q}} \tanh^{-1}\left(\frac{k_1}{\sqrt{q}}\right) \tag{18}$$

where $q = 4\,a\,k_1 k_2 + k_1^2$ for convenience in handling the equation. This equation describes the *course* of the reaction $S \rightleftarrows P + A$ under the new illumination.

2. The situation, though somewhat more complicated algebraically, is entirely analogous to the one described in detail in the previous section. Equations (17) and (18) contain the same variables, I, t, and x, as before, and the procedure for calculating corresponding values of x from I and t is also the same. Arbitrary numerical values may be assumed for the constants m and k_2, whereas a is made equal to 100 per cent. Let us put $k_2 = 0.001$ as before, and $m = 0.0001$. This value of m is $\frac{1}{100}$ of the value used in the preceding section, simply to make the numerical results come to about the same order of magnitude as before, since the presence of $a = 100$ in equation (13) multiplies the velocity by 100. Otherwise the values are purely as arbitrary as before, and in no way affect the results of the calculations.

Using these magnitudes for the constants m and k_2 we compute the values of x_o for the stationary state for the series of adapting intensities. The results are given in Column 3 of Table III and of Table IV, representing the experiments in Series 1 and Series 2, respectively.

TABLE III.

Calculation of Residual P and A Present at Adaptation, and of Fresh P and A for Stimulation. Series 1.

Adapting intensity.	Stimulating plus adapting intensity.	x_o	t_o	t_e	t_f	x_f	x_e
meter candles	*meter candles*	*per cent*	*sec.*	*sec.*	*sec.*	*per cent*	*per cent*
0.00	694.4	0.00	0.00	0.40	0.40	2.72	2.72
0.976	695.4	3.08	0.46	0.66	1.12	7.43	4.35
2.57	697.0	4.94	0.72	0.70	1.42	9.40	4.46
8.78	703.2	9.19	1.38	0.74	2.12	13.73	4.54
27.78	722.2	15.33	2.34	0.87	3.21	20.21	4.88
73.04	767.4	23.62	3.63	0.97	4.60	28.49	4.87
250.0	944.4	39.04	5.78	1.12	6.90	43.34	4.30
530.6	1,225.0	51.00	6.98	1.51	8.49	55.33	4.33

TABLE IV.

Calculation of Residual P and A Present at Adaptation, and of Fresh P and A for Stimulation. Series 2.

Adapting intensity.	Stimulating plus adapting intensity.	x_o	t_o	t_e	t_f	x_f	x_e
meter candles	*meter candles*	*per cent*	*sec.*	*sec.*	*sec.*	*per cent*	*per cent*
0.00	2,778	0.00	0.00	0.34	0.34	9.09	9.09
0.976	2,779	3.08	0.11	0.46	0.57	14.69	11.61
5.20	2,783	7.15	0.26	0.58	0.84	20.85	13.70
27.78	2,806	15.33	0.59	0.65	1.24	29.10	13.77
69.25	2,847	23.07	0.93	0.72	1.65	36.64	13.57
250.0	3,028	39.04	1.68	0.86	2.54	51.38	12.34
530.6	3,309	51.00	2.28	1.12	3.40	63.02	12.02

The corresponding values of t_o, the initial moment in stimulation, are then derived from equation (18), and are tabulated in Column 4 of the two tables. To these are added the sensitization or exposure periods t_e found in Column 5, which give the values of t_f in Column 6.

By means of equation (18) the final concentrations x_f of P and A are then computed; the results are in Column 7. If from these values of x_f are subtracted those of x_o in Column 3, we get x_e in the last column, as representing the amount of P and A freshly formed during the exposure to the stimulating light.

This series of equations and the resulting calculations, in order to be consistent, should prove that the amount of freshly formed P and A necessary to produce stimulation in *Mya* is constant, regardless of the intensity of adaptation. Considering all the factors involved in their derivation, it is apparent that these values of x_e are practically constant for each series of experiments. This is particularly evident if we neglect the values at adaptation to zero intensity, which we shall consider presently. It is therefore apparent that of the two, the "constant quantity" assumption is strikingly more consistent with the experimental data, and that in terms of it the experiments find a rational and simple explanation. It should therefore be adopted as the basis on which the reaction $S \rightleftarrows P + A$ works to produce photosensory stimulation in *Mya*.

3. It is obvious from an examination of the values of x_e that the variation in its magnitude is not haphazard but regular in both sets of experiments. Beginning with zero intensity, the values rise to a maximum and then decrease. Although the data show a reasonably constant value of x_e, these regular variations are not without significance, particularly since their existence may be correlated with another process in the sense organ.

The reaction time of *Mya* is composed of a sensitization or exposure period and a latent period (Hecht, 1918–19, *a*). The former is represented by the reaction $S \rightleftarrows P + A$, the products of which catalyze the latent period reaction $L \rightarrow T$ (Hecht, 1918–19, *b*). When a sufficient amount of T has accumulated, an afferent impulse proceeds to the nervous system and the retraction reflex is initiated. The velocity of this latent period reaction, $L \rightarrow T$, is a linear function of the concentration of P and A, so that for very small exposures the latent period varies inversely as the exposure. Ordinarily, however, the exposure is prolonged until the velocity of $L \rightarrow T$ is maximal, and the latent period is minimal, because the necessary exposure is only a small portion of the reaction time. Therefore the latent

period is constant, and the only variable in the reaction time is the sensitization period.

Considered in detail, this situation explains most of the variation in the value of x_e. When a very small exposure is given, the velocity of $L \rightarrow T$ is very slow, and a long latent period will be required to produce enough T to set off the nervous impulse. If a longer exposure is given, the velocity of $L \rightarrow T$ is increased, and the necessary latent period will be shorter. For a still longer exposure, a still shorter latent period will result; and if the exposure is continued indefinitely, as in the present experiments, the velocity of $L \rightarrow T$ becomes greater and greater. But since T has begun to form from the first moment, even at the slow rate, enough of it to initiate the nervous impulse will accumulate before the calculated amount is formed during the shorter latent period resulting from the increased amount of P and A present at the end of the exposure. The reaction time is, therefore, strictly a minimum period comprising a minimum exposure and a minimum latent period. When the exposures are small, as they usually are, the latent period is measurably constant in comparison, it being often ten or even a hundred times longer than the exposure. When, however, the exposure must be prolonged because of the low speed of the reaction $S \rightleftharpoons P + A$, the latent period will become longer than at the shorter reaction times.

Such is the case in these experiments, because the exposures cover a comparatively large range. To facilitate the experimental procedure and the calculations, I have taken the latent period as constant. It is undoubtedly slightly longer when the animals are light adapted than when they are dark adapted. The difference, though small in comparison to the length of the latent period, is large in comparison to the exposure. Therefore, the values of x_e will be correspondingly lower at zero intensity adaptation than at all other intensities, and in general less at the lower adaptation intensities than at the higher, which is true experimentally. The whole matter will probably become cleared up with further experimentation in which the exposure time will be kept constant, and the *intensity* necessary for a minimum response will be measured at a series of adaptation intensities.[3]

[3] It is possible that the characteristic shape of the curve in Fig. 4 is also in part determined by these changes in latent period. This will be apparent on further experimentation along the lines suggested.

Regardless of these minor variations, however, the results of these experiments point unequivocally to the adoption of the hypothesis that a given amount of freshly formed P and A is necessary to produce a given stimulation in *Mya*, no matter what the previous conditions of stimulation or adaptation have been.

VI.

The fact that a consistent analysis of the experimental data has resulted from the application of the reversible reaction $S \rightleftharpoons P + A$ as the chemical mechanism of photosensitivity in *Mya*, lends a distinct support to that hypothetical mechanism. As a working basis, it has already been satisfactorily applied to the treatment of a large number of experiments not only with *Mya*, but with the human eye as well. It is simple and concrete, and what is perhaps more significant, experimental consequences from it are easily deducible in mathematical form.

There are, however, certain cautions that should be mentioned, so that the ideas underlying the mechanism will not be misunderstood. The chemical equations expressing the different parts of the sensory process are based on kinetic, not stoichiometric data. Therefore it would be just as accurate to say that, instead of the photosensitive reaction being

$$\underset{\text{"dark"}}{\overset{\text{light}}{S \rightleftharpoons P + A}} \qquad (1)$$

it is really

$$\underset{\text{"dark"}}{\overset{\text{light}}{\begin{matrix} S \rightarrow P + A + B \\ S \leftarrow P + A + C \end{matrix}}} \qquad (19)$$

In this, C is a substance or a source of energy necessary for the dark reaction, but present in excess in the sense organ. The first process, equation (1), is completely reversible, whereas the second, equation (19), is only pseudoreversible. It is quite probable that the second

set of equations is nearer reality. Since, however, both require identical mathematical treatments, I use the symbols of the completely reversible reaction, because they are simpler in conception.

Similar cautions apply to the relation between the photosensitive reaction and the latent period process $L \rightarrow T$. Calling this relation a process of catalysis (Hecht, 1918–19, *b*) is merely a non-committal way of describing the situation. The *fact* is that the velocity of the latent period process is a linear function of the exposure, or in symbols, that the velocity of $L \rightarrow T$ is a linear function of the decomposition products of S. Whether this involves P or A or both, or perhaps B—which appears in the pseudoreversible reaction—it is impossible to say at present. There is some material or energetic linkage between the two reactions, symbolically written $L \parallel P + A \parallel \rightarrow T$, which results in the linear relation between them. Further than that it is better not to speak dogmatically until additional data become available.

VII.

1. As a result of these experiments, there are certain conclusions that are of general interest. A sense organ, from its very nature, is a dual mechanism; on the one hand it connects with the environment, while on the other it connects with an afferent nerve. Such a duality may be attained by a single chemical reaction in which the environment alters a sensitive substance, and this altered substance then acts on the nerve ending to produce an afferent impulse.

In *Mya*, all the evidence has shown that this duality is accomplished by means of two chemical reactions. The reception of the stimulus is performed by the reaction $S \rightleftarrows P + A$. The decomposition products of S are, however, not the substances which furnish the *Anlass* for the afferent impulse. This is done by the thermolabile material T, formed as a result of the process $L \rightarrow T$, a reaction which occurs only in the presence of freshly formed P and A. The accumulation of T in the region of the nerve endings results in some physicochemical change. The substance T may actually unite with something in the nerve fiber, or what is more likely, its heaping up in the sense organ may result in a difference of potential between the sense cell and the nerve ending of sufficient magnitude to initiate a sensory impulse.

We may express the idea of this duality of the sense organ as follows:

$$\text{Light} \rightarrow \boxed{S \rightleftarrows P + A;\ L \parallel P + A \parallel \rightarrow T} \rightarrow \text{Impulse}$$

in which the reactions in the rectangle take place inside the sense cell.

2. Considered from this point of view, there are two very important points which appear with regard to the properties of a sensory system such as that of *Mya*. When the light is first turned on, the freshly formed P and A cause the reaction $L \rightarrow T$ to proceed, and a sensory impulse results, giving a response on the part of the animal. The reaction, $S \rightleftarrows P + A$, however, quickly comes to equilibrium, and no fresh P and A are formed. The condition, asfar as the reaction $L \rightarrow T$ and the production of an afferent impulse are concerned, is therefore equivalent to one in which there is no light shining on the animal at all. This fact is demonstrated by the condition of the animal, which to all appearances looks and behaves as if it were no longer stimulated by the light. Its retracted siphon becomes expanded, and its water current continues in the same normal manner after sustained exposure to all intensities from zero to the highest sunlight illuminations. This, I take it, is adaptation.

One important point shown by our analysis is that this adaptation is entirely contained in the sense organ. More than that, even in the sense organ it takes place in the receiving end only. It is the reaction $S \rightleftarrows P + A$ which is balanced at the stationary state, and not the second reaction $L \rightarrow T$. Between the nervous system of the animal and the outside environment there is interposed a sensory system which acts so as to transmit any rapid changes in the environment, but which hides completely any long maintained conditions.

This becomes even more evident at different levels of adaptation. In order to produce a given effect, the amount of P and A, and therefore the amount of T formed is constant, no matter what the adapting light happens to be. Note carefully that the actual amount of outside light necessary to produce this constant amount of P and A varies strikingly. We speak of this as a variation in sensitivity. The impulse from the sense organ, however, is the same. The sense organ in this way acts as a buffer, in that it changes these large amounts of environmental disturbance into a given constant disturbance which

constitutes the impulse for a given sensory effect. The sensory system is therefore not merely the traditional receptor system, but is as well a protecting layer which stabilizes and buffers the relation between the nervous system and the environment.

3. From all this it must be clear that adaptation to sustained illumination is a sensory phenomenon purely. The second point of significance with regard to the present data is that they indicate the automatic nature of the processes involved. Not only does adaptation take place in the sense organ, away from nervous activity, but it is actually determined by the outside source of stimulation. The equation which describes the situation in the sense cell during adaptation is

$$\frac{m\,I}{k_2} = \frac{x^2}{a - x} \tag{17}$$

which we derived in Section V of this paper. In this equation the only two variables are the intensity I and the concentration x of the decomposition products P and A, and therefore of the concentration of the sensitive substance S. Of these two variables it is the intensity I which changes independently, and with it change the concentrations of S, P, and A. Given a value of I, and the equation is at once determined. In short, adaptation is a phenomenon which is controlled not by the animal, but by the light. We have heretofore applied the term adaptation in the wrong place as far as sensory adaptation is concerned. We have assumed that the animal adapts itself to the light, whereas, once given the sensory mechanism, we should say that the light adapts the animal to itself.

4. Among the many relations which an organism maintains with its environment, none would seem so purposeful as its adaptation to a sustained change in the environment. This kind of adaptation clearly belongs to those activities of an animal which have been called its "behavior," and as such, has been considered as remote from analysis in terms of matter and energy as it is possible to get.

Because of this appearance of purpose, sensory adaptation has been used in the study of animal behavior as a tool with which to prove the presence of a "higher behavior" in animals. It would be beside the point to make specific references to literature of this nature.

Mya constitutes one of the very few closely studied instances of the sensory behavior of an animal. And from this work it has been apparent for some time, and more so now, that such a use of adaptation is wholly unwarranted. The apparently complicated relation which we call sensory adaptation is not so much an activity of the organism as a whole, as a series of changes induced in the outermost shell of the organism, and controlled by the very outside environment to which the adaptation is supposedly directed from the inside.

SUMMARY.

1. Experiments are described which measure the sensitivity of animals exposed to continued illumination to which they have become adapted. It is shown that the amount of outside light energy necessary to stimulate an adapted animal increases with the intensity of the adapting illumination.

2. The data are analyzed quantitatively in terms of the reversible reaction $S \rightleftharpoons P + A$ shown previously to account for the photic sensitivity of these animals. This analysis demonstrates that, though the amount of incident energy necessary for a minimal response varies with the adapting intensity, the actual amount of photochemical decomposition required to set off the sensory mechanism is a constant quantity.

3. The ability of these animals to come into sensory equilibrium with any sustained illumination is accounted for quantitatively by the presence of a stationary state in the reversible photochemical reaction $S \rightleftharpoons P + A$ during which the concentrations of the three components are constant.

4. It is shown that the concentrations of these substances at the stationary state are automatically controlled by the outside intensity. Therefore, given the sensory mechanism as a basis, the adaptation of the animals to light and the consequent changes in sensitivity, are determined entirely by the light to which the animals are exposed.

5. Because of the properties of the stationary state, and of the constancy of photochemical decomposition for a minimal effect, it is suggested that the sensory system is not only the traditional receptor system, but is also a protecting layer which stabilizes and buffers the relation between the nervous system and the environment.

BIBLIOGRAPHY.

Hecht, S., (*a*) Sensory equilibrium and dark adaptation in *Mya arenaria*, *J. Gen. Physiol.*, 1918–19, i, 545; (*b*) The nature of the latent period in the photic response of *Mya arenaria*, 657; (*c*) The effect of temperature on the latent period in the photic response of *Mya arenaria*, 667; (*d*) Time and intensity in photosensory stimulation, 1920–21, iii, 367; (*e*) The nature of foveal dark adaptation, 1921–22, iv, 113.

Luther, R., and Weigert, F., Ueber umkehrbare photochemische Reaktionen in homogenen Systeme. Anthracen und Dianthracen, *Z. physik. Chem.*, 1905, li, 297.

THE PHOTOCHEMICAL BASIS OF ANIMAL HELIOTROPISM.

By JOHN H. NORTHROP and JACQUES LOEB.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

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I.

One of us published in 1888 a preliminary notice,[1] followed by a monograph in 1889,[2] in which it was shown by experiments chiefly on insects that the motion of animals to a source of light is not due to an attraction of the animals by the light, but is due to an automatic orientation of the animal by the light, as a consequence of which the animal is forced to move to the source of light. It was shown that if there is only one source of light, certain animals are automatically oriented by the light in such a way that their heads are turned towards the source of light and that their planes of symmetry are turned into the direction of the rays of light. In that case, the animal is automatically compelled to move to the source of light.

The action of light was ascribed by Loeb to a chemical effect on the retinæ or some sensitive spots of the skin. If there is only one source of light, and the plane of symmetry of the animal goes through the source of light, the symmetrical eyes (or the symmetrical photosensitive elements of the skin) are struck by the light at the same angle, and the intensity of illumination is the same for symmetrical elements. When, however, symmetrical elements of the eyes (or skin) of the animal are no longer struck at the same angle by the source of light, *e.g.* when the animal is illuminated sidewise, the intensity of illumination by the source of light is no longer the same for the symmetrical retinæ or other symmetrical photosensitive elements, and the animal is automatically compelled to change the direction of its motion in such a way that its plane of symmetry is again brought

[1] Loeb, J., *Sitzungsber. phys.-med. Ges. zu Würzburg*, 1888.

[2] Loeb, J., Der Heliotropismus der Tiere und seine Uebereinstimmung mit dem Heliotropismus der Pflanzen, Würzburg, 1889.

into the direction of the rays of light. As soon as this happens, the animal will be compelled again to move in a straight line towards the source of light. This was explained by Loeb in the following way. The tension of the symmetrical muscles of the locomotor organs is influenced by the light in a similar way to that in which it is influenced by the action of gravity on the internal ear in the higher animals. As long as the rate of photochemical change in symmetrical parts of the photosensitive organs is the same, the tension of symmetrical muscles in the locomotor organs—legs, wings, or swimmerets—will be affected in the same way and the animal will continue to move in as straight a line as the imperfections of its locomotor apparatus permit. When, however, the rate of photochemical change is no longer the same in symmetrical elements of the eye or skin, the tension of the symmetrical muscles of the locomotor apparatus will no longer be the same and the direction of the motion of the animal will be automatically changed. This change may either bring the head towards the source of light or away from the source of light. When the head is automatically turned towards the source of light, we speak of positive heliotropism, and when the head is turned automatically away from the light, we speak of negative heliotropism. As soon as the plane of symmetry falls again into the direction of the rays of light, the symmetrical muscles again assume the same tension and the animal is automatically forced to move in a straight line either towards or away from the source of light. What appeared to the earlier investigators as a mysterious attraction of the animals by the light (in the case of positively heliotropic animals) or as a mysterious flight from the light (in the case of negatively heliotropic animals) thus turned out to have been only a case of automatic orientation of the animal due to a photochemical effect on the retina or other photosensitive elements of the surface of the animal. The phenomenon was thus amenable to a purely physicochemical analysis, according to the principles of photochemistry.

II.

In an address delivered in 1911, Loeb suggested that the physicochemical law determining these automatic heliotropic reactions of animals was Bunsen and Roscoe's photochemical law, whereby the

photochemical effect, E, is proportional to the product of the intensity, I, into the duration, t, of illumination.

$$E = K \cdot I \times t$$

where K is a constant.[3] The idea was tested and confirmed in his laboratory by Ewald[4] on the heliotropic orientation of *Daphnia*, by Loeb and Ewald,[5] by Loeb and Wasteneys[6] on the heliotropic curvature of *Eudendrium*, by Loeb and Northrop[7] on the larvæ of *Balanus*, and by Patten,[8] in Parker's laboratory, on the heliotropic orientation of the larvæ of the blowfly.[9] It is intended to show in this paper that the law holds also for the orientation of the horseshoe crab (*Limulus*).

Young specimens of the horseshoe crab about 15 cm. in length were used. In each experiment one specimen was put into a square aquarium with straight glass walls (Fig. 1), and its orientation under the simultaneous influence of two lights, a and b, was ascertained. In order to permit the exact measurement of the orientation of the animal with respect to the lights, it was necessary to limit the extent of motion of the animal, without limiting its freedom of assuming a definite orientation with regard to the two lights. For this purpose the tail of the animal was fastened with the loop of a short cotton thread to a nail fixed in the bottom of the aquarium so that the animal could turn without friction in any direction without being able to move beyond the distance of the length of the thread from the nail. Loeb had found that the larvæ of *Limulus* are positively heliotropic in cold, and negatively heliotropic in warmer water.[10] This seems to be the case for these older specimens too, which were mostly negative at room temperature, though not all the specimens reacted to light. A small number, ten out of forty-eight specimens, could be used for the tests to be described. That not all reacted may or may not have been due

[3] Loeb, J., The mechanistic conception of life, Chicago, 1912.

[4] Ewald, W. F., *Science*, 1913, xxxviii, 236; *Z. Sinnesphysiol.*, 1914, xlviii, 285.

[5] Loeb, J., and Ewald, W. F., *Zentr. Physiol.*, 1913–14, xxvii, 1165.

[6] Loeb, J., and Wasteneys, H., *J. Exp. Zool.*, 1917, xxii, 187.

[7] Loeb, J., and Northrop, J. H., *Proc. Nat. Acad. Sc.*, 1917, iii, 539.

[8] Patten, B. M., *J. Exp. Zool.*, 1914, xvii, 213.

[9] All the work on Loeb's theory of tropisms is discussed in his book, Forced movements, tropisms, and animal conduct, Philadelphia and London, 1918.

[10] Loeb, J., *Arch. ges. Physiol.*, 1893, liv, 81.

to their exhausted condition, since they had been kept in the laboratory under unfavorable conditions.

Two Mazda incandescent lamps of equal intensity (determined with the Lummer-Brodhun photometer) were used. They were placed as indicated in Fig. 1. In order to allow the exact measurement of the

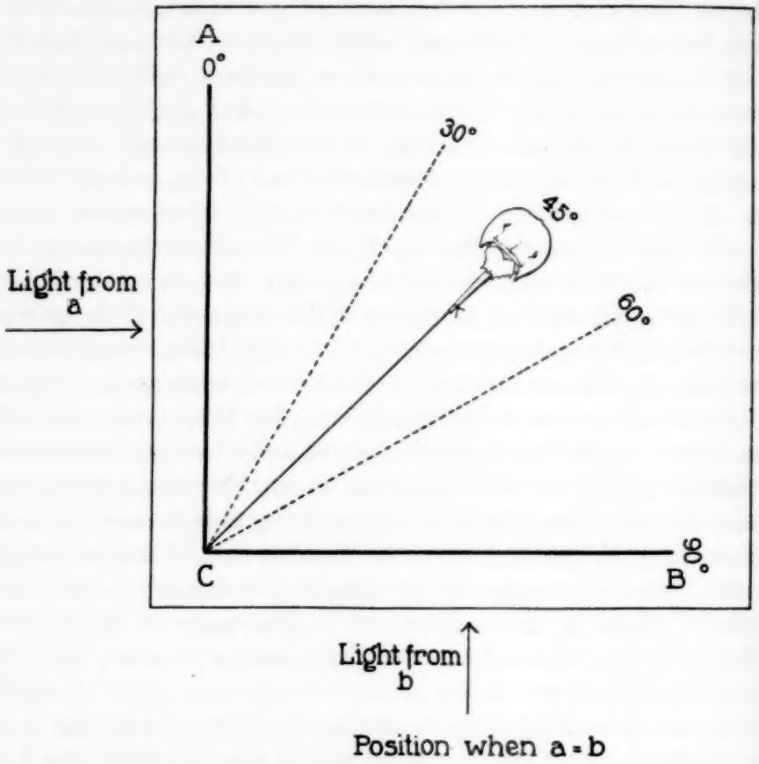

FIG. 1. Orientation of the negatively heliotropic crab with respect to two lights, *a* and *b*, when the intensity of the two lights is equal.

orientation of the plane of symmetry of the animal with respect to the two lights, a quadrant, with the nail as center, marked on the bottom of the aquarium, was divided into sectors of 5° each, the zero line being parallel to one illuminated side of the aquarium, the 90° line to the other illuminated side (Figs. 1 and 2).

When only one light was used, *e.g.* light *a*, the negatively heliotropic animal oriented itself in the path of the light, namely the line CB (90°), with its tail toward the light; when only light *b* was turned on, the animal came into the stationary position of 0°. In each case the animal put its plane of symmetry into the direction of the rays of

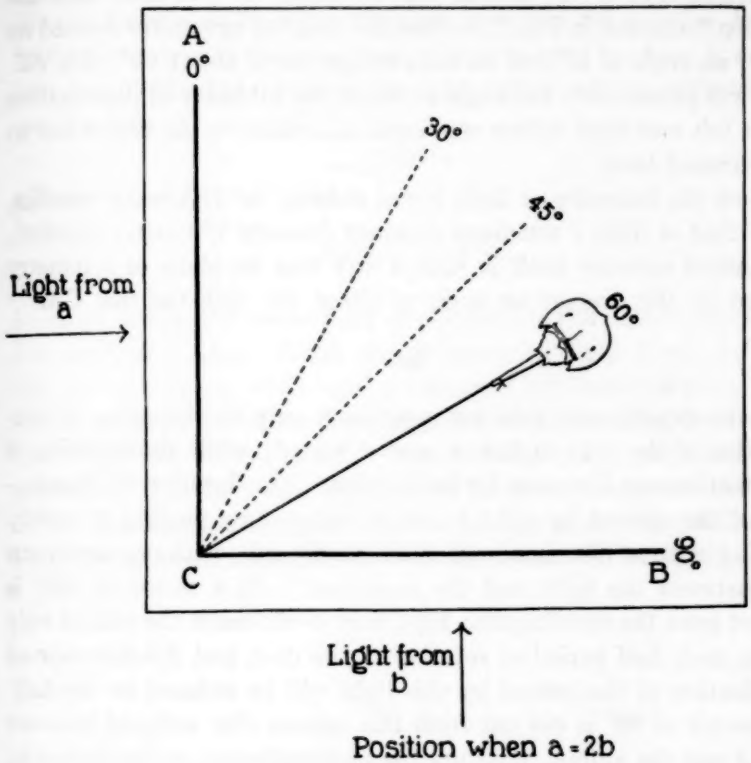

FIG. 2. Orientation of the negatively heliotropic crab with respect to two lights, *a* and *b*, when the intensity of *a* is twice as great as the intensity of *b*.

light. When both lights were turned on simultaneously and when the intensity of the two lights was equal, the plane of symmetry of the animal coincided with the angle of deflection of 45° from the line AC (Fig. 1). During the whole time the animal made active swimming motions, trying to escape, and these motions kept it in the stationary position of about 45°, as shown in Fig. 1.

In all the following experiments the intensity of light *a* remained stationary, namely 150 meter candles, while the intensity of light *b* was changed by increasing its distance from the aquarium.

When light *b* was put so far away from the aquarium that its intensity was only 75 meter candles, *i.e.* only half the intensity of *a*, the orientation of the animal was different. It put itself into the position indicated in Fig. 2, so that its plane of symmetry formed no longer an angle of 45° but on the average one of about 60° with AC. This was presumably the angle at which the intensity of illumination of the left and right retinæ was equal according to the cosine law to be discussed later.

When the intensity of light *b* was reduced to 37.5 meter candles, while that of light *a* remained constant (namely 150 meter candles), the animal oriented itself in such a way that its plane of symmetry formed on the average an angle of about 68° with the line CA.

III.

In the experiments thus far mentioned only the intensity of illumination of the two lights *a* and *b* varied, while the duration of illumination was the same for both lights. The duration of illumination of the animal by light *b* can be reduced by putting a rapidly rotating opaque disc (made of thick cardboard), with one sector cut out, between the light and the aquarium.[11] If a sector of 180° is cut out from the rotating disc, light from *b* will reach the animal only during each half period of rotation of the disc, and the duration of illumination of the animal by this light will be reduced to one-half. If a sector of 90° is cut out from this opaque disc and put between light *b* and the animal, the duration of illumination of the animal by light *b* is reduced to one-fourth. Now if the general law of photochemistry, namely the Bunsen-Roscoe law, determines the heliotropic orientation of the animal, the animal should always put its plane of symmetry into the line bisecting the angle ACB, whenever the product of intensity into duration of illumination is the same for the two lights *a* and *b*.

[11] This method of varying the duration of illumination had been used by Ewald, Patten, and Loeb and Northrop in the papers referred to.

In all the following experiments the intensity of light *a* was kept constant, namely 150 meter candles, while the intensity and duration of illumination of *b* were changed.

When the intensity of light *b* was increased to 300 meter candles, while the intensity of light *a* remained 150 meter candles, it was found that the animal put its plane of symmetry into the line 45° (Fig. 1), bisecting angle ACB, when the open sector of the rotating disc was 180°. In this case the duration of illumination by light *b* was cut into two, so that the product of duration into intensity of illumination was for light *a*

$$150 \times t$$

and for light *b*

$$300 \times \frac{t}{2} = 150 \times t$$

In other words, the orientation of the animal was determined by the Bunsen-Roscoe law. When the intensity of light *b* was raised to 600 meter candles, while light *a* remained 150 meter candles, it was found necessary to reduce the open sector of the rotating disc between light *b* and the animal to 90°, to force the animal to put itself in the position of Fig. 1, where its plane of symmetry bisected the angle ACB. In this case the product of duration into intensity of illumination was again the same for the two lights, namely for light *a*

$$150 \times t$$

and for light *b*

$$600 \times \frac{t}{4}$$

which again conforms with the Bunsen-Roscoe law.

IV.

We have seen that when no rotating disc is used, *i.e.* when the duration of illumination is the same for both lights, the animal puts its plane of symmetry into an average angle of about 60° with the line AC when the ratio of the intensity of *a* to *b* is as 2:1. Now the law of Bunsen and Roscoe demands that this orientation should always occur when the ratio, product of $I \times t$ for light *a* over that of the

same product for light b, is as 2: 1, regardless of whether the intensity alone or the duration alone or both are changed. This was found to be the case.

When the intensity of both lights was 150 meter candles, the duration of illumination of light b had to be cut exactly to one-half with the aid of the rotating disc to force the crab to put its plane of symmetry into the position shown in Fig. 2, where the plane of symmetry of the animal forms an angle of 60° with the line AC.

In another series of experiments the intensity of light b was increased to 300 meter candles, while that of light a remained 150 meter candles. In this case it was found necessary to cut down the time of illumination of light b to one-fourth (by using a rapidly rotating disc with a sector of 90° cut out) in order to compel the animal to put its median plane at an angle of 60° with the line AC. In both of these experiments the value of the illumination product, $I \times t$, for a and b, was as 2: 1.

Finally it was possible to show that in order to compel the animal to put its plane of symmetry into a line forming an angle of about 68° with the line AC, it was necessary to make the value of the product $I \times t$ for light b one-fourth of the value of the product $I \times t$ for light a. Thus light a had the intensity of 150 meter candles and no disc was put before this light. When the light b had the intensity of 75 meter candles, the open sector of the rotating disc between light b and the animal had to be 180° in order to force its plane of symmetry into forming an angle of 68° with the line AC. In this case the ratio of the illumination products $I \times t$ for the two lights a and b was

$$Ia \times ta : Ib \times tb = 150 \times 1 : 75 \times \tfrac{1}{2} = 4 : 1$$

When the intensity of light b was 150 meter candles, the open sector of the rotating disc had to be 90° to compel the animal to put its plane of symmetry into a line forming an angle of 68° with AC. In this case the ratio of illumination product for a and b was again 4: 1.

$$Ia \times ta : Ib \times tb = 150 \times 1 : 150 \times \tfrac{1}{4} = 4 : 1$$

These results leave no doubt that the heliotropic orientation of the horseshoe crab is determined by a photochemical reaction which follows the law of Bunsen and Roscoe.

V.

The results of our experiments are tabulated in Table I. For the explanation of this table the following data should be remembered. The intensity of light a (Ia) was always constant, namely 150 meter candles, and so was the duration of illumination t of light a, which was 1, since no rotating disc was put in front of this light. The illumination product for light a, $Ia \times ta$, was therefore always $150 \times t$. The intensity of light b (Ib) varied, as the first vertical column of the table indicates, between 0 and 600 meter candles. The relative duration of illumination, tb, varied between 0.25 and 1.0. This time is the fraction of the period of rotation during which the light could reach the animal.

The third column of the table gives the illumination product for light b, namely $Ib \times tb$, and the fourth column gives the ratio of the illumination products of light b and light a, namely

$$\frac{Ib \times tb}{Ia \times ta}$$

The last vertical row of the table gives the average angle of the plane of symmetry of the animal with the line AC (Figs. 1 and 2).

The table shows that the value of this angle is the same for the same value of

$$\frac{Ib \times tb}{Ia \times ta}$$

In order to give the reader an idea of the individual variations, the value of the angle of orientation of the animal is given for each of the individual specimens of *Limulus* tried.

Ten individuals were found to orient themselves to the light, practically all being negatively heliotropic. Each animal is numbered and some animals were used repeatedly on different days; *e.g.*, Animals 1, 6, and 9.

Each individual figure is the average of ten consecutive measurements taken within 1 minute. The animal made constant swimming motions but was prevented from making any progressive motion, being held by the thread attached to the nail (Figs. 1 and 2).

All the variations in intensity and duration of illumination of light b recorded in the vertical Column 1 were tried for each animal in rapid

succession. There were variations in the orientation of different individuals but these were chance variations which were equal and opposite for different individuals. The average angular deflection of the animal from the path of light *b* (the line AC) is given in the last vertical column of Table I.

TABLE I.

Angular Deflection of Limulus from Path AC of Light b.

Intensity of light *a* constant = 150 meter candles.
Duration of illumination by light *a* constant = 1.
Intensity × duration of light *a* constant = 150.
Intensity and duration for light *b* variable.

Ib	tb	$Ibtb$	Ratio $\frac{Ibtb}{Iata}$															Average angular deflection.	
meter candles				7	5	2	3	4	1	1	6	6	12	9	9	9	18		
150	1.0	150	1.0	40	46.5	45	46	46.5	46	47	50	42	50	43	41	43	42	45.1	±0.6
300	0.5	150	1.0	45	42		44	42.5	43	48	57	41	43	41	43	46	49	45.2	±0.8
600	0.25	150	1.0	45	45	42	44	44.5	48	50	52	40	49	46	46	46	42	45.7	±1
75	1.0	75	0.5	60	55	65	70	69	72	54	70	46	58	58	53	56	50	59.7	±2
150	0.5	75	0.5	61	60	59	71	70	69	58	63	55	62	63	58	57	51	61.2	±2
300	0.25	75	0.5	60	58		68	69	68	56	61	53	50	63	64	58	51	60.0	±2
37.5	1.0	37.5	0.25	70	61	70	74	78	79	66	78	61	71	64	67	69	63	68	±2
75	0.5	37.5	0.25	67	63		70	73	72	59	75	57	70	67	68	60	57	66	±2
150	0.25	37.5	0.25	70	60	64		74	79	65	78	66	71	69	70	68	53	68	±2
0	1.0	0	0	89.5	88	92	91	90	88	87	92	86	91	91	90	87		89.4	±0.6

VI.

An animal is therefore automatically oriented by the light in such a way that the illumination products $I \times t$ are the same for the two eyes. When only one source of light exists, this is always the case when the plane of symmetry of the animal falls into the direction of the rays of light, *i.e.* goes through the source of light. When two lights of equal intensity act simultaneously on the animal, this happens when the plane of symmetry of the animal bisects the angle that its head (or rather that point of its head which we may assume to represent Hering's imaginary cyclopic eye, substituted for the retinæ of the animal) forms with the two lights.

In both cases the symmetrical points of the eyes or skin of the animals are struck by the rays of light at the same angle. The importance of this angle was pointed out by Loeb in his first publications referred to above. The photochemical significance of this angle lies in the cosine law which states that the intensity of illumination of a surface element varies with the cosine of the angle of incidence of the light. If this angle be α, then the expression for the orienting effect, E, of light becomes

$$E = K \cdot I \times t \times \cos \alpha$$

Now Loeb had also insisted on the fact that symmetrical elements of the eyes or of the photosensitive parts of the skin of the animal are not only equal morphologically but also chemically; *i.e.*, the same illumination will produce equal chemical effects on two symmetrical elements, but not, as a rule, on dissymmetrical elements. This explains why, when only one source of light exists, the animal is automatically oriented by the light in such a way that symmetrical elements of the retinæ are struck by the light at the same angle, α. In this case the rate of photochemical change in both eyes is the same when I and t are the same. The heliotropic orientation of animals by light is, therefore, such that the value of the product

$$I \times t \times \cos \alpha$$

is the same for both eyes (or any other symmetrical photosensitive elements of the animal).

It is obvious what must happen when the value of $I \times t$ is no longer the same in both eyes. If α remains the same for both eyes, the rate of photochemical change in the two retinæ will be different when $I \times t$ is different for the two eyes, and hence the symmetrical muscles of the locomotor organs will undergo a different degree of tension, a fact to which we shall return presently. If the value of $I \times t$ is greater in the left than in the right eye (as in Fig. 2), the equality of photochemical effect in the two eyes can be restored only when the value of cos α becomes greater for the right eye (b) than for the left eye (a), $\cos \alpha_b > \cos \alpha_a$ (Fig. 2), or, in other words, the animal will be automatically compelled to put its plane of symmetry into such a position that it forms a greater angle than 45° with the line AC which

is nearer the stronger light *a*. Through this change of position α becomes smaller in the right than in the left eye and hence cos α becomes greater in the right than in the left eye. This is rendered evident in Diagram 3. It would be even possible to calculate beforehand the ratio of the two angles for each case if only the surfaces of the retinæ were plane and parallel. According to Loeb's theory the animal must put its plane of symmetry into such a position that the product of

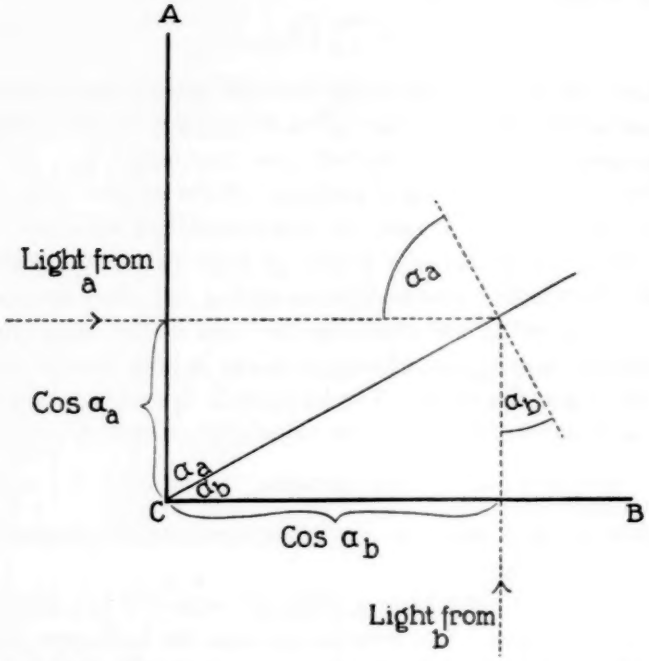

FIG. 3. Diagram illustrating the variations of the intensity of illumination with the cosine of the angle of incidence.

$I \times t \times \cos \alpha$ is the same for the two eyes. If now $I \times t$ is twice as great for the left as for the right eye,

$$2 \times \cos \alpha_a \text{ (left eye)} = \cos \alpha_b \text{ (right eye)}$$

In other words, the cosine of the angle of incidence of light on the left retina, cos α_a, must be only one-half of the cosine of the angle of incidence in the right eye, cos α_b. The animal must change its position

in such a way that the angle of incidence, α_a, for the stronger light on the left retina is greater than the angle of incidence, α_b, for the weaker light on the right retina (Fig. 3), a result which is actually observed. On account of the curvature of the retinæ of the horseshoe crab and on account of the fact that it possesses in addition to two lateral, also median eyes, it is impossible to test this postulate of Loeb's theory quantitatively for *Limulus*. Patten's[8] observations on the heliotropic orientation of the larvæ of the blowfly may be used as an approximate quantitative confirmation of this part of the theory. Qualitatively, however, the theory is supported by all the observations of the heliotropic reactions of animals.

VII.

Finally the question arises as to why it happens that the animal is automatically oriented in such a way that $I \times t \times \cos \alpha$ has the same value for the symmetrical eyes or the symmetrical elements of its photosensitive surface. The answer to this was given by Loeb by the suggestion that the products of decomposition of the photosensitive substance act on the sensory nerves connected with the eye and thereby influence the tension of the muscles. The connection between the rate of photochemical change and muscular reaction of the animal has been investigated by Hecht[12] from a physicochemical viewpoint in the case of reactions of *Mya* and *Ciona* to light, and he has been able to define the nature of the reaction. On the basis of his measurements it is safe to say that the rate of decomposition of a photosensitive substance in the eye or skin of an animal influences the tension of muscles. Now experiments on the influence of gravity on the ear of higher animals leave no doubt that the tension of the symmetrical groups of muscles which move the eyes or head and subsequently the whole body to the right or left is acted upon as a unit; and also that these two units are linked with the two symmetrical halves of the photosensitive or the geotropically sensitive elements of eyes or internal ear respectively. Experiments have shown that upon asymmetrical illumination animals are either compelled to move to that side where the product $I \times t \times \cos \alpha$ is greater or where it is smaller;

[12] Hecht, S., *J. Gen. Physiol.*, 1919–20, ii, 229, 337; 1920–21, iii, 367, 375.

in the former case we speak of positively heliotropic animals, in the latter of negatively heliotropic animals. In the case of positively heliotropic animals the tension of muscles turning the head towards that source of light increases with the value of $I \times t \times \cos \alpha$, while in the case of negatively heliotropic animals the tension of the same muscles is lowered. The reason for this difference is not yet known. When the eyes of a positively heliotropic animal are struck asymmetrically by light the rudder action of the swimmerets, turning the head toward the source of light, will be stronger than that of their symmetrical antagonists, and the animal will automatically deviate towards the source of light until its plane of symmetry is again in such a position that the value of $I \times t \times \cos \alpha$ is again the same for both eyes. In that case the influence of the light on symmetrical muscles is the same and the animal will continue to move in that direction. This part of Loeb's theory has been put to a test by a number of writers, among others Holmes,[13] Garrey,[14] Minnich,[15] and Cole,[16] and proved to be correct. Of the many experiments of these authors special attention may be called to the fact that when one eye is covered, the animal moves constantly in a circle around a source of light, the open eye facing the center of the circle when the animal is positively heliotropic. This is a consequence of the fact that in such a case the rudder action of the symmetrical organs of locomotion is no longer the same but is stronger in those muscles which turn the head of the animal towards the source of light. Moreover, it was shown by Garrey[14] and Cole[16] that the curvature of the circle in which such animals move becomes the greater the greater the intensity of light. Cole[16] has shown that the relation between intensity of light and curvature is logarithmic in agreement with the Weber-Fechner law. It follows further that the heliotropic orientation can only be expected in a moving animal, since when the animal rests there can only be a difference in the relative tension of symmetrical muscles. This may result in peculiar postures which have been observed by Garrey,[14] but it is not necessary that they should result in a definite orientation

[13] Holmes, S. J., *J. Comp. Neur. and Psych.*, 1910, xx, 145.
[14] Garrey, W. E., *J. Gen. Physiol.*, 1918–19, i, 101.
[15] Minnich, D. E., *J. Exp. Zool.*, 1919, xxix, 343.
[16] Cole, W. H., *J. Gen. Physiol.*, 1922–23, v, 417.

of the plane of symmetry with reference to the light. Thus heliotropic animals at rest may occupy any position with reference to the light, while when they move they will be automatically oriented on account of the rudder effect of the symmetrical legs or swimmerets of the animal.

Differences in the heliotropic sensitiveness of different animals will probably be found to depend upon the relative mass and nature of the photosensitive substance in their eyes or skin, in the relative quantity of decomposition products required to cause reflexly a change in the tension of their muscles, and in the nature of the nervous connection of the eyes with the symmetrical muscles of locomotion.

VIII.

SUMMARY.

1. Experiments on the heliotropic orientation of *Limulus* were made which confirmed Loeb's photochemical theory of animal heliotropism proposed first in 1888 and 1889 in experiments on insects, and later in experiments on other forms of animals.

2. It is shown that these animals are oriented by light in such a way that the product

$$I \times t \times \cos \alpha$$

is the same for the symmetrical photosensitive elements of the eyes or the skin, where I is the intensity of the light, t the duration of illumination, and α the angle of incidence of the light at the surface element of the photosensitive organ.

3. When this equation holds, the products of decomposition by light must be the same in symmetrical elements of the eyes or skin, and the influence of these products of decomposition on the tension of symmetrical muscles of the locomotor organs of the animal must be the same. As a consequence the animal must move in the path of light, either to or from the source of light.

HOMOSTROPHIC REFLEX AND STEREOTROPISM IN DIPLOPODS.

By W. J. CROZIER and A. R. MOORE.

(From the Departments of Zoology and Physiology, Rutgers College, New Brunswick.)

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I.

In former papers (1, 2) by one of us, it has been shown in annelids that reflex orientation of the head segments in response to bending of the posterior part (3) is a reaction mediated by a definite neuromuscular mechanism. This reaction has been called the homostrophic reflex (1). The receptors of this reflex lie in the muscle sheath and are distributed throughout the length of the animal; the efferent nerves, on the other hand, have their origin in a limited number of the anterior ganglia, 15 to 20 in *Lumbricus*, 2 to 3 in *Nereis*. The afferent impulses travel forward by way of the ventral nerve cord. Since bending of the head has no effect on the course of backward locomotion, the reaction shows definite polarity. Attention has been called to the fact that certain vertebrates, namely fishes and rabbits, show a muscle tension reflex similar to the homostrophic reflex of annelids. Consequently, it is of interest to find how widespread is the occurrence of this type of regulatory reaction among the invertebrate phyla.

Among the arthropods, most hexapods and crustaceans present the difficulty that the body is incapable of extensive lateral movement. With the larvæ of *Tenebrio molitor* it is possible to obtain some evidence of the homostrophic reflex, but the results are not clear because of the variable tension of the abdominal musculature. Material suitable for the demonstration of the reflex is available among the diplopods, *Julus venustus*, *Parajulus pennsylvanicus*, and *Polydesmus*.

The locomotion of these animals has been studied by Clementi (4), who also considers the "spiral reflex" characteristic of them.

The "spiral reflex" involves chiefly curvature in the dorsoventral plane. He and other observers seem not to have noticed the effects of lateral tension.

II.

It can easily be shown that the effects of unilateral tension on the position of the head and the direction of locomotion in diplopods correspond perfectly with those already described for annelids. The experiments are best made in the dark room under red light, and with animals from which the antennæ have been removed. The latter operation is not necessary, but seems to increase the precision of the reaction by removing a source of tactile excitation.

If the animal is put down with the body curved in the shape of a crescent, when locomotion starts the head end moves so as to direct the course in a line very nearly parallel with the position of the tail. Records were obtained by allowing the animal to walk over smoked paper (Fig. 1, *a* and *b*). When a specimen is creeping actively, gentle bending of the tail to one side results in accurate orientation of the head region in a line parallel to that made by the tail (Figs. 2 and 3). This reflex orientation of the head also may be induced in quiescent animals by passive unilateral tension of the posterior region of the body. The zone of the body in which tension must be applied to be effective lies between the fourth or fifth segment from the anterior end and the fourth segment from the posterior end. The experiment cannot be made at the extreme limits of the body because the few segments at either end cannot be bent. It is probable, therefore, that the receptors are distributed throughout the body as in annelids.

If the ventral nerve cord is cut at any level, by means of ventral incision with a sharp needle, then the position of the head of the diplopod is not affected by bending the animal posterior to the cut. This proves that the afferent impulse is propagated over the ventral nerve cord.

Removal of the head leaves a preparation which may remain alive in a moist atmosphere for a week and which exhibits characteristic movements, even of locomotion, but no trace of homostrophic orientation is elicitable. The brain, therefore, plays the same necessary part in the homostrophic reflex of the diplopods as do the brain and

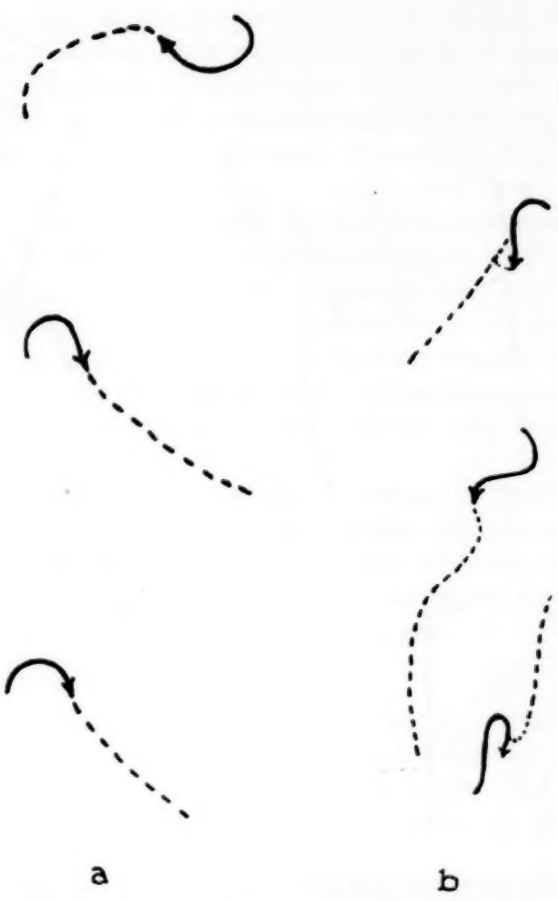

FIG. 1. *a*. Showing how the direction of progress is influenced by unilateral tension, when *Julus* is put down on a surface with the body curved. In these three cases the homostrophic bending is not so great as in some instances.

b. Instances of homostrophic orientation of the head of *Julus*, more precise than in *a*. Here the phenomenon is not obscured by the retrograde wave of body straightening.

The full line indicates initial position of the body; the dotted line shows subsequent course.

(In Figs. 1, 2, and 3 the paths indicated have been traced from records made by the diplopods upon smoked paper.)

FIG. 2. The change in path of progression induced by lateral displacement of the tail. The dotted line shows the path after sideward bending of the tail.

FIG. 3. The homostrophic orientation of the head following lateral contact with an obstacle (x). The fact that the reflex may be somewhat delayed increases the appearance of "intelligent" pursuit of a straight path.

adjacent ventral ganglia in *Nereis*, and as do the anterior 15 to 20 ganglia in *Lumbricus*. It is of interest that in diplopods the first three segments behind the head which homologize with the thoracic region of the insects (4), morphologically and physiologically, do not contain the origin of the motor neurons of the homostrophic reflex.

In order to determine whether the receptors of the reflex lie in the muscles of the legs or in the body wall, the legs were clipped from the median region for about one-fourth the length of the animal. In a second experiment all the legs were removed. In the latter case, of course, no locomotion was possible, only bending of the body. In either case the homostrophic reflex follows appropriate displacement of the tail. Hence the receptors for the reflex are in the body wall.

In backward locomotion, whether of normal or decapitated specimens, enforced displacement of the anterior end does not affect the direction of locomotion. This is identical with the corresponding phenomenon in annelids, and proves the existence of similar polarity in the mechanism.

III.

There are two types of reflex response which may modify or even mask the homostrophic reflex in diplopods. First, it is frequently seen, especially when the animal is moving rapidly, that the tail may be brought into alignment with the anterior part by a sort of retrograde wave of straightening.

In the second place, stereotropism is effective in masking the homostrophic reflex, since during the time a part of the animal's body remains in unilateral contact with a surface, the head will turn in the direction of the region of contact. This may be shown in the following way. The diplopod is allowed to crawl along in contact with the edge of a triangular glass plate. Upon reaching the corner, the head deflects toward the side which is still partially in contact with the glass (Fig. 4, *a*, *b*). If, now, the glass be removed while the body of the animal is still bent, the existing curvature will cause homostrophic bending of the head (Fig. 4, *c*), although previously it was unable to do so. The case of stereotropism just described is of especial interest for two reasons.

First, the response occurs only while the exciting force is acting; *i.e.*, while a part of the body is in contact with a surface. The reaction is therefore not the result of *change*, but rather of *constant action* of the physical force.

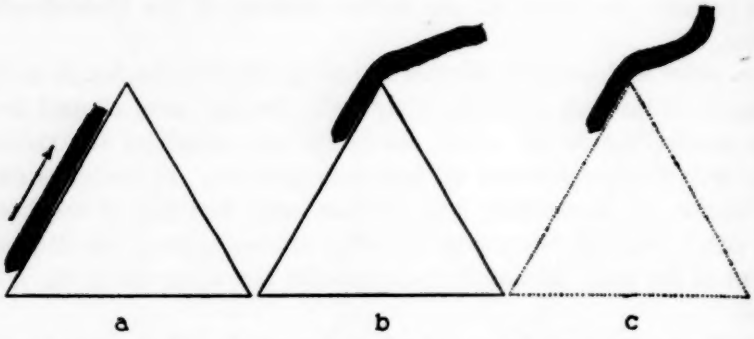

FIG. 4. Stereotropic orientation of the anterior end of *Julus*, induced by unilateral contact with a thick glass plate; *a* and *b* show successive positions. During stereotropic orientation the homostrophic reflex is inhibited, as is demonstrated by the immediate effect of removing the glass plate shown at *c*, where the head bends to a course parallel with the position of the tail.

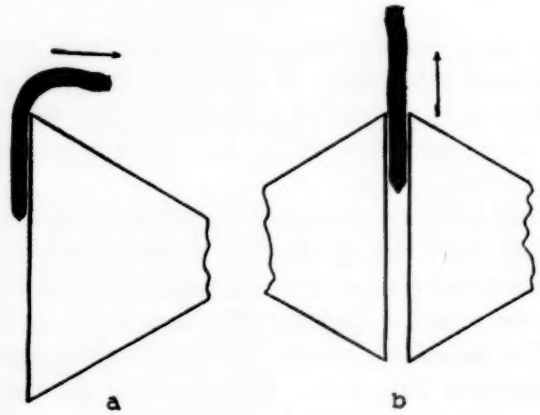

FIG. 5. Proof of the vector character of stereotropism. In *a* is shown the effect of unilateral contact upon the path of *Julus* creeping beyond the corner of the glass plate. In *b* is shown the path of *Julus* emerging from the zone of equal bilateral contact with two such plates. Balanced stimulation results in absence of bending.

Second, the response shows a vector character in that the direction of locomotion is determined by unilateral contact (Fig. 5, *a*). It should be possible, then, by producing equal contacts on the two sides, to cause the animal to creep in a straight line as it leaves the zone of contact. In fact this is precisely what happens. A diplopod is allowed to crawl between two parallel glass plates so placed that the edges just touch the animal on either side. When the animal crawls out into the open field it does not bend to either side but pursues a straight course (Fig. 5, *b*).

This phenomenon is analogous to the reaction of the tube feet of starfish to two points of contact (5). The tube feet between the two points bend at right angles to a line joining the points. This relationship of direction of movement to two sources of stimulation in tropisms was first worked out for heliotropism (6, 7). It has been proven by a number of workers that an animal which is heliotropic responds to two lights of equal intensity by moving along a path which bisects the angle formed by the lights with the organism. The reaction of a diplopod to contact stimulation of the two lateral surfaces of the body is strictly comparable to the reaction of a heliotropic organism exposed to two lights of equal intensity. In each case the organisms behave according to the law of the parallelogram of forces when the two forces are equal.

IV.

SUMMARY.

1. With suitable arthropods, such as the diplopods, it can be shown that body orientation following passive unilateral tension involves the homostrophic reflex. The phenomenon is exhibited when the animal is quiescent and during forward locomotion, but nothing of the sort appears in backward locomotion.

2. Receptors for the homostrophic reflex are in the body wall and are distributed throughout the length of the animal.

3. The effector nerves take their origin from the ganglia of the head alone.

4. The diplopods are stereotropic, the head turning toward the side in contact with a solid surface only as long as some part of the

body maintains contact. Under suitable conditions stereotropism may mask the homostrophic reflex.

5. When a diplopod is in contact with two lateral surfaces of equal extent the path upon emergence is a straight one, conforming to the law of the parallelogram of forces.

BIBLIOGRAPHY.

1. Moore, A. R., *J. Gen. Physiol.*, 1922–23, v, 327.
2. Moore, A. R., *J. Gen. Physiol.*, 1922–23, v, 451.
3. Morgulis, S., *J. Comp. Neur. and Psych.*, 1910, xx, 616.
4. Clementi, A., *Zool. Jahrb., Abt. Allg. Zool.*, 1911–12, xxxi, 277.
5. Moore, A. R., *J. Gen. Physiol.*, 1921–22, iv, 163.
6. Loeb, J., Studies in general physiology, Chicago, 1905, i, 82.
7. Patten, B. M., *J. Exp. Zool.*, 1914, xvii, 213.

THE STABILITY OF BACTERIAL SUSPENSIONS.

VI. The Influence of the Concentration of the Suspension on the Concentration of Salt Required to Cause Complete Agglutination.

By JOHN H. NORTHROP.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, March 20, 1923.)

It has been shown in the previous papers of this series[1] that the addition of electrolytes to suspensions of bacteria (*Bacillus typhosus* and the bacillus of rabbit septicemia) affects two properties of the suspension. First, the potential difference between the organisms and the liquid is changed, and second, the cohesive force (the force with which the organisms adhere) is affected. Low concentrations of electrolytes affect primarily the potential, and higher concentrations (more than 0.1 N) primarily the cohesive force. It has further been shown that, if the cohesive force is not affected, agglutination occurs whenever the potential is reduced below about 13 millivolts. If the cohesive force is decreased, this critical potential is lowered and in high concentration of salt no agglutination occurs, although the potential may be too small to measure. In the case of suspensions which had been treated with immune serum, the cohesive force is not affected by any concentration of salt, and agglutination occurs whenever the potential is decreased to less than 13 millivolts. Suspensions sensitized with immune serum therefore behave just as oil emulsions studied by Powis[2] and present a much less complicated phenomenon. The point of complete agglutination in this case is therefore a convenient method of determining the salt concentration required to decrease the potential to 13 millivolts.

[1] Northrop, J. H., *J. Gen. Physiol.*, 1921–22, iv, 629. Northrop, J. H., and De Kruif, P. H., *J. Gen. Physiol.*, 1921–22, iv, 639, 655. De Kruif, P. H., and Northrop, J. H., *J. Gen. Physiol.*, 1922–23, v, 127, 139.

[2] Powis, F., *Z. physik. Chem.*, 1915, lxxxix, 186.

EXPERIMENTAL PROCEDURE.

Suspensions of *Bacillus typhosus* were used. They were prepared by washing in distilled water as described in a previous paper,[1] and then suspended in $\frac{1}{20}$ of the volume of the original broth culture. 0.5 cc. of a powerful antityphoid horse serum was then added to 500 cc. of this suspension and dilutions were made in distilled water so as to furnish suspensions of relative concentrations of 1, 3, and 9. The amount of serum added was in excess of that needed to cause agglutination with the minimum amount of NaCl. The amount of electrolyte added with the serum is negligible.

1 cc. of the suspensions was then added to 1 cc. of the salt solutions as noted in the tables, the tubes were allowed to stand at 20°C. for 18 hours, and the degree of agglutination was read. The sharpest point is that marked as complete (C). This represents the condition in which the suspension is completely settled and the supernatant liquid perfectly clear. +++ was recorded when the supernatant liquid was slightly cloudy.

The results of the experiments are shown in Tables I and II. Table I shows that the concentration of NaCl, $NaNO_3$, Na_2SO_4, KCl, LiCl, NaBr, NH_4Cl, $BaCl_2$, and $MgSO_4$ required to cause complete agglutination is the same irrespective of the concentration of the suspension. The potential measurements reported in a preceding paper show that none of these salts reverses the sign of the charge. The results of the different salts are not comparable as they were made at different times and in some cases with different suspensions. The result of importance here is the comparison of the degree of agglutination of the various concentrations of suspension with the same salt. The series with any one salt were made at the same time and under as closely comparable conditions as possible.

The results of the experiments with those salts that had been found to reverse the sign of the charge on the organism (except for $ZnSO_4$) are given in Table II. The table shows that in each case the amount of salt increases in proportion to the concentration of suspension, as nearly as can be determined from the experimental data. The results with $LaCl_3$ are especially interesting, since there are two zones of agglutination. The one in the lower concentration represents the

TABLE I.

Effect of Concentration of Sensitized Typhoid Suspension on Concentration of Salt Required to Cause Complete Agglutination.

Salt.	Relative concentration of suspension.	Degree of agglutination in the following concentration of salts; equivalents per liter.					
		0.05	0.025	0.0125	0.00625	0.0031	0.0015
NaCl	1	C.	C.	+++	++	+	+
	3	C.	C.	+++	++	+	+
	9	C.	C.	+++	+++	++	+
$NaNO_3$	1	C.	C.	+++	++	+	−
	3	C.	C.	+++	++	+	+
	9	C.	C.	+++	++	−	+
Na_2SO_4	1	C.	C.	+++	++	+	−
	3	C.	C.	+++	++	+	+
	9	+++	C.	C.	++	+	+
KCl	1	C.	C.	+++	++	++	+
	3	C.	C.	+++	++	++	+
	9	C.	+++	+++	+++	++	+
LiCl	1	C.	C.	C.	++	+	+
	3	C.	C.	C.	++	+	+
	9	C.	C.	C.	++	+	+
NaBr	1	C.	C.	C.	++	+	+
	3	C.	C.	C.	++	+	+
	9	C.	C.	C.	++	+	+
NH_4Cl	1	C.	C.	+++	++	+	+
	3	C.	C.	+++	++	+	+
	9	+++	C.	+++	++	+	+
$BaCl_2$	1	C.	C.	C.	+++	++	+
	3	C.	C.	C.	+++	++	+
	9	C.	C.	C.	+++	++	+
$MgSO_4$	1	C.	C.	C.	+++	++	+
	3	C.	C.	C.	+++	++	+
	9	C.	C.	C.	+++	++	+

point at which the sign of the charge changes. The agglutination in high concentration is at the point at which the charge is again lowered, but does not become reversed.

TABLE II.

Effect of Concentration of Sensitized Typhoid Suspension on the Concentration of Salt Required to Agglutinate.

Electrolyte.	Concentration of suspension.	Degree of agglutination in the following concentrations of salts; equivalents per liter.														
		0.51	0.256	0.128	6.4×10^{-3}	3.2×10^{-3}	1.6×10^{-3}	8×10^{-4}	4×10^{-4}	2×10^{-4}	1×10^{-4}	5×10^{-5}	2.5×10^{-5}	1.28×10^{-5}	6.4×10^{-6}	3.2×10^{-6}
$HgCl_2$	1						C.	C.	C.	C.	C.	C.	+++	++		
	3						C.	C.	C.	C.	C.	+++	++	+		
	9						C.	C.	C.	C.	+++	++	+	−		
$ZnSO_4$	1						C.	C.	C.	C.	+++	+	+			
	3						C.	C.	+++	+++	++	+	−			
	9						C.	+++	++	++	+	+	−			
Copper acetate	1			+++	++	+	−	−	+	+++	C.	C.	C.	++	+	−
	3			++	+	+	+	++	C.	C.	C.	+++	++	+	+	−
	9			++	+	++	+++	C.	C.	C.	+++	++	++	+	+	−
$LaCl_3$	1	C.	C.	+++	++	+	+	−	−	−	+	+	C.	+	−	
	3	C.	C.	+++	++	+	+	−	+	+++	C.	+++	+	+	−	
	9	C.	C.	+++	++	++	++	+	C.	+++	+++	+	+	+	−	
HCl	1							−	−	−	−	+	+	C.	−	−
	3							−	−	−	+	C.	+	−	−	−
	9							−	−	++	C.	+	+	−	−	
H_2SO_4	1							−	−	−	+	C.	+	−		
	3							−	−	+	C.	+	−	−		
	9							+	+	C.	+	−	−	−		

It will be noted that there is no distinct valency effect as far as the relation of the suspension concentration to the agglutinating concentration of salt is concerned.

The influence of the chemical nature of the ion, however, is apparent. The alkali and alkali earths, which are inert as regards proteins and do not form complex ions easily with ammonia or amino groups, do not combine chemically or reverse the sign of the organisms. The heavy metals and hydrogen ion, which are known to form complex ions readily with ammonia and amino groups in general, do reverse the sign of charge and behave as though they combined chemically with the suspension.

SUMMARY.

1. The concentrations of various salts required to agglutinate different concentrations for a suspension of typhoid bacilli sensitized with immune serum have been determined.

2. The electrolytes may be divided into two classes; (1) those with which the concentration required to agglutinate is independent of the concentration of the suspension; and (2) those with which the agglutinating concentration increases in proportion to the concentration of the suspension.

3. The salts comprised under (1) do not reverse the sign of the charge of the suspension.

4. The salts of Class (2) (with the exception of $ZnSO_4$) do reverse the sign of the charge.

COMPARATIVE STUDIES ON RESPIRATION.

XXV. The Action of Chloroform on the Oxidation of Some Organic Acids.

By GEORGE B. RAY.

(*From the Laboratory of Plant Physiology, Harvard University, Cambridge.*)

(Received for publication, March 19, 1923.)

In a previous paper (1) it has been shown that *Ulva* which has been killed by drying and subsequently treated with H_2O_2 and $Fe_2(SO_4)_3$ may be made to produce CO_2 after the manner of the living organism, and that the rate of production of CO_2 may be affected by the presence of chloroform.

In order to throw some light on the mechanism of these reactions experiments similar to those described in the preceding paper of this series were undertaken, in which various organic acids were used in place of killed *Ulva*.

The first substance studied was oleic acid. In this experiment, the results of which are shown in Fig. 1, 50 cc. of the acid were placed in the reaction flask of the apparatus, and the rate of production of CO_2 was determined. Section AB of the curve shows that some CO_2 is produced. The time necessary to change the indicator tube from pH 7.78 to 7.36 was about 10 minutes. The addition of 25 cc. of approximately molar H_2O_2 caused the rate to increase. This is shown in Section BC. When $Fe_2(SO_4)_3$ is added to the mixture of acid and peroxide, one finds another increase, as is shown in Section CD.

Since colloidal metals are also known to act as oxygen catalysts, a solution of colloidal silver was also tested. This was prepared in the following manner. A direct current of 110 volts was allowed to pass between two silver electrodes placed in distilled water. The resulting dark brown solution was used as the catalyst. Its action is shown in Section CE of Fig. 1. As may be seen the effect is similar to that of iron. Since such solutions are difficult to standardize it was decided to use iron in further experiments.

Tannic acid was the next substance tested; it was found to act in the same manner as oleic acid. In order to show the relation of the components of the mixture to the production of CO_2, varying concentrations of the constituents were placed in the reaction flask and the rate determined after 1 hour.

The composition of the mixtures is represented according to the method of Roozeboom (2) by means of an equilateral triangle (Fig. 2),

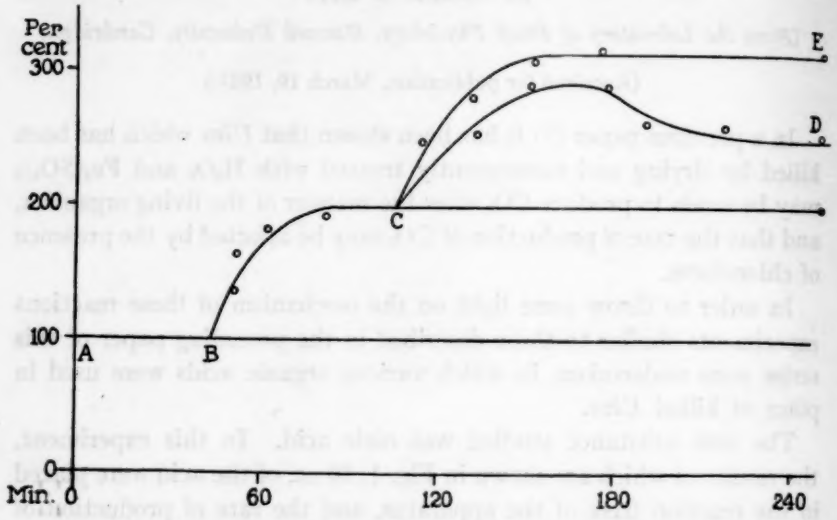

FIG. 1. The effect of the addition of H_2O_2 and the subsequent addition of either $Fe_2(SO_4)_3$ or colloidal silver to oleic acid. Section AB represents the rate of production of CO_2 by oleic acid alone (arbitrarily called 100 per cent); Section BC is the effect of the addition of 3 per cent H_2O_2; Section CD the effect of 25 cc. 0.0004 M $Fe_2(SO_4)_3$; and Section CE the addition of 25 cc. of a colloidal silver solution. The points represent the average of five experiments. The probable error of the mean is less than 5 per cent of the mean.

the apices of which represent (*A*) 100 per cent tannic acid (molar), (*B*) pure H_2O_2 (molar), (*C*) pure $Fe_2(SO_4)_3$ (0.0002 molar). Points on the sides of the triangle represent mixtures of the two solutions indicated by the adjacent apices. Therefore point G would represent 75 per cent H_2O_2 and 25 per cent $Fe_2(SO_4)_3$; H represents 50 per cent of each; I, 25 per cent of the peroxide and 75 per cent of the iron salt. In the same way P indicates 75 per cent H_2O_2 and 25 per cent tannic

acid; E, 50 per cent of each; F, 25 per cent of the peroxide and 75 per cent of the acid. Points in the interior of the triangle represent mixtures of all three of the constituents. Thus O would indicate a mixture consisting of 50 per cent iron since it is on line HK; it is also on line PI which represents 25 per cent H_2O_2, and on line F which shows it to have 25 per cent tannic acid. In this manner the relation of the constituents of the system to each other may be defined. The ordinates, which represent the reciprocal of the time of production of a unit amount of CO_2, are plotted perpendicularly to the plane of the triangle. This gives a solid, as shown in Fig. 3.

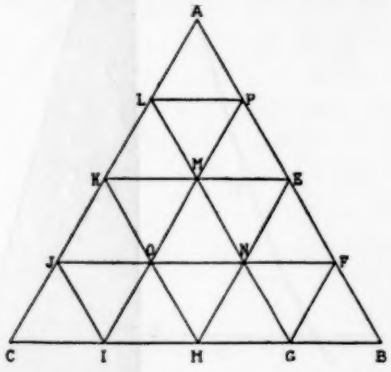

FIG. 2. The composition of the mixtures of tannic acid, H_2O_2, and $Fe_2(SO_4)_3$ according to Roozeboom's method. For explanation see text.

From these data it will be seen that tannic acid alone produced no CO_2, but on the addition of H_2O_2 a small amount is given off. $Fe_2(SO_4)_3$ alone does not oxidize the tannic acid, but when added to a mixture of H_2O_2 and tannic acid considerable CO_2 is produced. The rate of production of CO_2 is proportional to the amount of iron. The temperature at which these experiments were conducted was $20° \pm 2°$C. The probable error of the mean is less than 5 per cent of the mean.

Succinic acid and its unsaturated homologues, fumaric and maleic acids, present an interesting group for experimentation. First of all, they differ from the preceding compounds inasmuch as they give true solutions. They also form a series in themselves since they comprise a saturated acid and the cis- and trans- forms of the cor-

responding unsaturated acids. They are especially interesting from the biological point of view since fumaric and succinic are found in muscle tissue, while maleic is not. Verkade and Söhngen (3) found

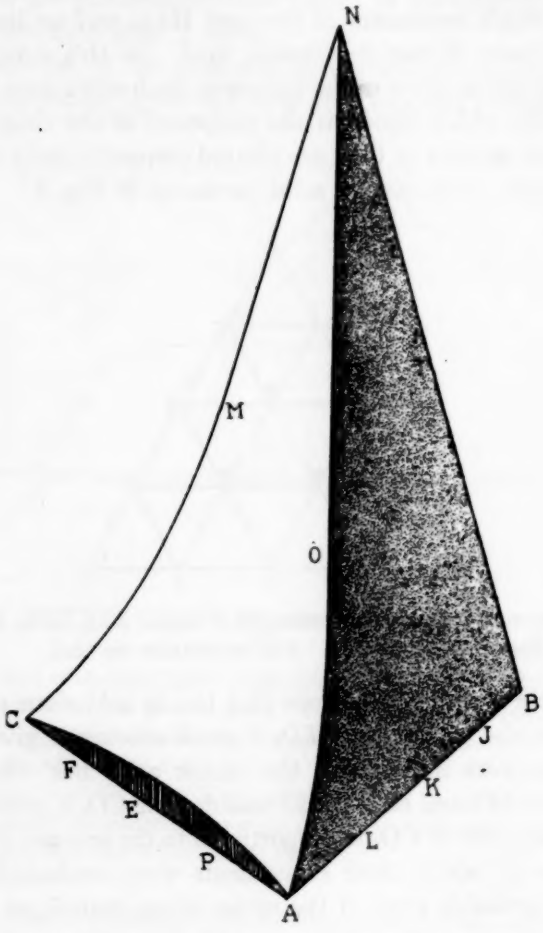

FIG. 3. The rate of production of CO_2 by the mixtures represented in Fig. 2.

that fumaric acid is attacked by *Aspergillus* and *Penicillium* in the presence of an excess of $CaCO_3$, while maleic is not. This was also found to be true of other cis- and trans- isomers.

Fig. 4 shows the results of these experiments in which the same technique was used as in the case of the previous experiments. It is interesting to note that the acids which occur in the organism

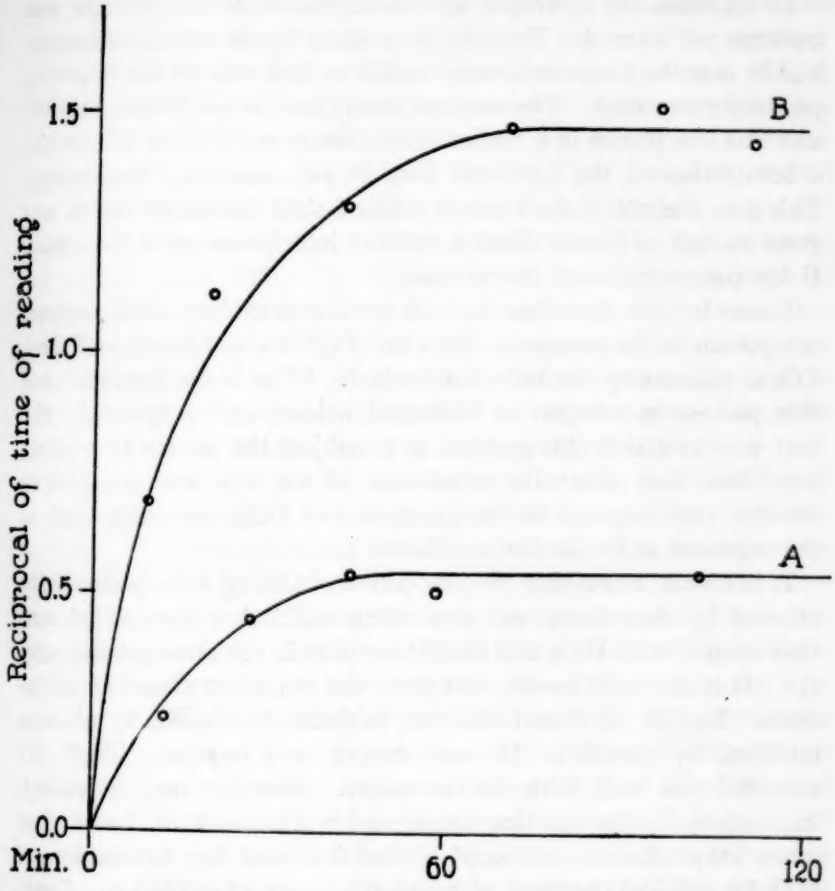

FIG. 4. The production of CO_2 by succinic (Curve A) and fumaric (Curve B) acids when treated with H_2O_2 and $Fe_2(SO_4)_3$. The curves are the average of five experiments. The probable error of the mean is less than 5 per cent of the mean.

are oxidized by H_2O_2 and $Fe_2(SO_4)_3$, while maleic acid did not produce sufficient CO_2 to be measured. These compounds were used in 0.2 M solutions or suspensions.

The following compounds were also tested and found to produce CO_2 in measurable quantities: pyrogallol, lecithin, cinnamic acid, hydrocinnamic acid, and glycocoll.

In all cases the hydrogen ion concentration of the mixture was between pH 3 and 4. This was determined by the use of indicators, and in case the compounds were turbid or dark colored the following procedure was used. The mixture was placed in a diffusion thimble and this was placed in a beaker of distilled water. After 1 hour the concentration of the hydrogen ions in the water was determined. This gave the pH of the reaction mixture since the buffer action was great enough to permit dilution without interference with the actual H ion concentration of the mixture.

It may be seen, therefore, that it is possible to oxidize certain organic compounds in the presence of H_2O_2 and $Fe_2(SO_4)_3$ and obtain sufficient CO_2 to measure by the indicator method. What is the significance of this process in relation to biological oxidations? Apparently the best way to attack this problem is to subject the system to various conditions that affect the respiration of the cell, and to observe whether their response (in the production of CO_2) resembles that of the organism under similar conditions.

It has been shown that the production of CO_2 by *Ulva* is decidedly affected by chloroform, and that tissue which has been killed and then treated with H_2O_2 and $Fe_2(SO_4)_3$ reacts in the same general way (1). It is also well known that narcotics can affect chemical oxidations. Bigelow (4) found that the oxidation of Na_2SO_3 by air was inhibited by narcotics. He used copper as a catalyst. Titoff (5) extended this work with similar results. Attention may be called, incidentally, to the fact that Bredig and von Berneck (6) found that ether, ethyl alcohol, and amyl alcohol inhibited the destruction of H_2O_2 by colloidal platinum, although this is not an oxidation. Centnerszwer (7) worked on the oxidation of phosphorus to phosphorus trioxide under the influence of steam and found this reaction to be affected by narcotics. Warburg (8) showed that the oxidation of oxalic acid by blood charcoal was arrested by the presence of members of the urethane series. Vernon (9) and Baer and Meyerstein (10) have shown that narcotics inhibit the action of tissue oxidases on organic compounds. Battelli and Stern (11) found that tissue

oxidases (they worked with muscle extract) oxidized pyrotartaric acid to maleic; this action was arrested by the action of an anesthetic.

If it is possible to vary the rate of production of CO_2 by means of the addition of an anesthetic, we may be able to gain an insight into the action of the narcotic on the respiratory system of the organism.

Fig. 5 shows the effect of various anesthetics on the rate of production of CO_2 by tannic acid when it is treated with H_2O_2 and $Fe_2(SO_4)_3$. The general technique of these experiments was as follows: A mixture of 50 cc. of 10 per cent tannic acid, 25 cc. of 3 per cent H_2O_2, and 25 cc. of 0.0004 M $Fe_2(SO_4)_3$ was placed in the reaction flask of the apparatus for measuring the rate of production of CO_2, and a current of air free from CO_2 swept through for 1 hour. Then a series of readings was made to determine the "normal" rate. For convenience this is called 100 per cent. Then the reagent was added and the rate was determined as often as possible until the reaction appeared to be over. The curves are the result of five experiments. The probable error of the mean is less than 10 per cent of the mean.

Curve A shows the effect of ether on the rate of production of CO_2. The amount of the anesthetic added was 10 per cent by volume. It will be seen that the effect of ether is to cause an increase in the rate of production of CO_2. In this connection it may be mentioned that Acton (12) found that ether would catalyze the oxidation of arsenious acid by air. Curve B shows the effect of the addition of 1 per cent chloroform (by volume). The effect in this case is to cause a decrease in the rate of production of CO_2. This is followed by an increase and then by a decrease. The effect of chloretone is shown in Curves C and D. Curve C is the effect of the addition of 5 per cent of a saturated solution of chloretone; Curve D is the effect of 10 per cent. As may be seen, both concentrations cause an increase in the rate of production of CO_2. Curve E shows the effect of adding 5 per cent of a 20 per cent solution of chloral hydrate. This also causes an increase in the rate.

It might be thought that the increases that have been observed in the preceding experiments were due to the oxidation of the anesthetic. That this is not the case is shown by the fact that in control experiments (without tannic acid) there was no production of CO_2.

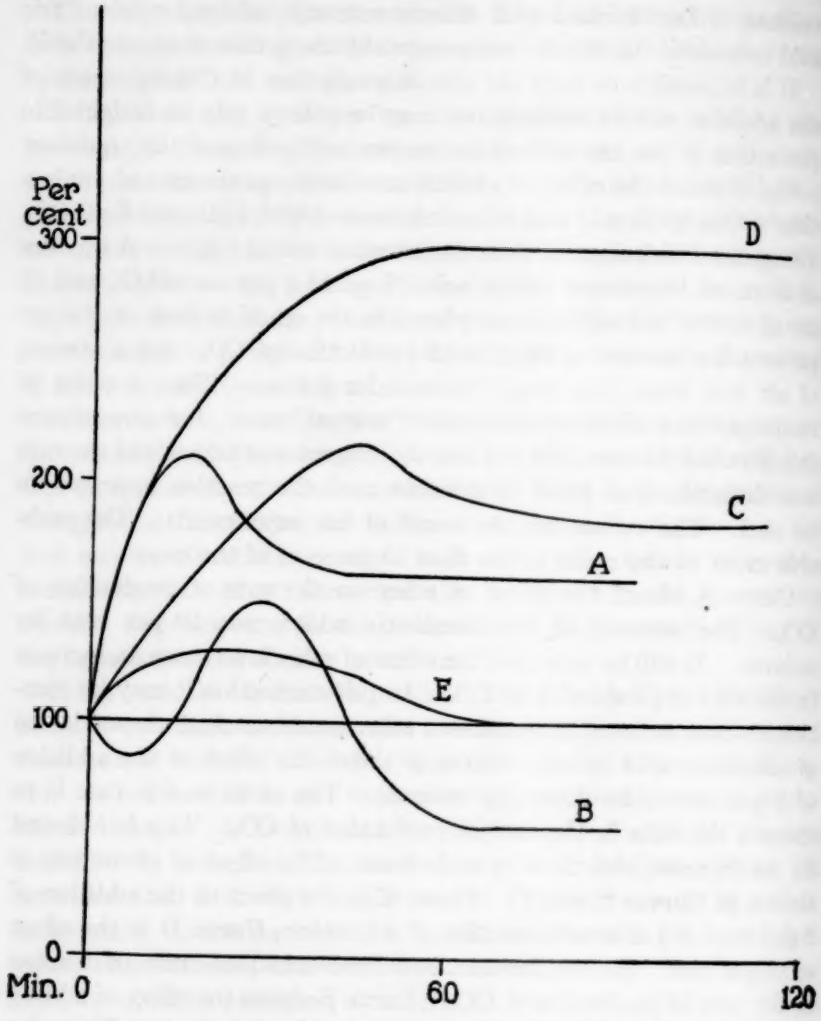

FIG. 5. The effect of various anesthetics on the rate of production of CO_2 by tannic acid in the presence of H_2O_2 and $Fe_2(SO_4)_3$. Curve A shows the effect of the addition of 10 cc. ether; Curve B of the addition of 1 cc. chloroform; Curve C of 5 cc. saturated chloretone; Curve D of 10 cc. of the chloretone solution; and Curve E the effect of 5 cc. of a 20 per cent solution of chloral hydrate. Each curve is the average of three experiments. The probable error of the mean is less than 10 per cent of the mean.

In planning further experiments it was thought desirable to concentrate effort on the study of one anesthetic. For this purpose chloroform was chosen since it is believed to be the most inert chemically.

Fig. 6 shows the effect of 1 per cent chloroform (by volume) on oleic and cinnamic acids. Curve A is the effect of the narcotic on oleic acid, and Curve B the effect on cinnamic acid. In both cases it is practically the same; there is a rise in the rate, which is followed by a fall. In the case of oleic acid, the mixture consisted of 50 cc. of the acid, 25 cc. of 3 per cent H_2O_2, and 25 cc. of 0.0004 M $Fe_2(SO_4)_3$. The mixture in the case of cinnamic acid was the same with the exception that 50 cc. of a 1 per cent suspension of cinnamic acid were used in place of the oleic acid.

Fig. 7 shows the effect of anesthetics on the rate of production of CO_2 by a mixture of fumaric acid, H_2O_2, and $Fe_2(SO_4)_3$. The amount of the acid was 50 cc. of 0.2 M. The concentrations of the other substances are the same, as in the preceding experiments. Curve A shows the effect of 1 per cent chloroform (by volume) and Curve B is the effect of 0.5 per cent. Both concentrations cause an increase in the rate, but only in the case of the greater concentration is there a drop below the normal rate. Curve C shows the effect of 10 per cent ether (by volume). The effect is almost the same as in the case of tannic acid; there is a continued rise in the rate for over an hour.

Experiments were also tried with hydrocinnamic acid, succinic acid, and glycocoll. In this case there was no change in the rate of production of CO_2. Apparently only those compounds that contain a double bond are affected.

In conclusion one might say that organic acids are oxidized by H_2O_2 and $Fe_2(SO_4)_3$, producing CO_2 at a rate that can be measured by the indicator method. In the case of unsaturated acids the rate of production of CO_2 may be affected by the addition of an anesthetic. Compared with the experiments reported in the preceding paper of this series on the effect of chloroform on *Ulva*, these results show a striking resemblance to the response of the organism.

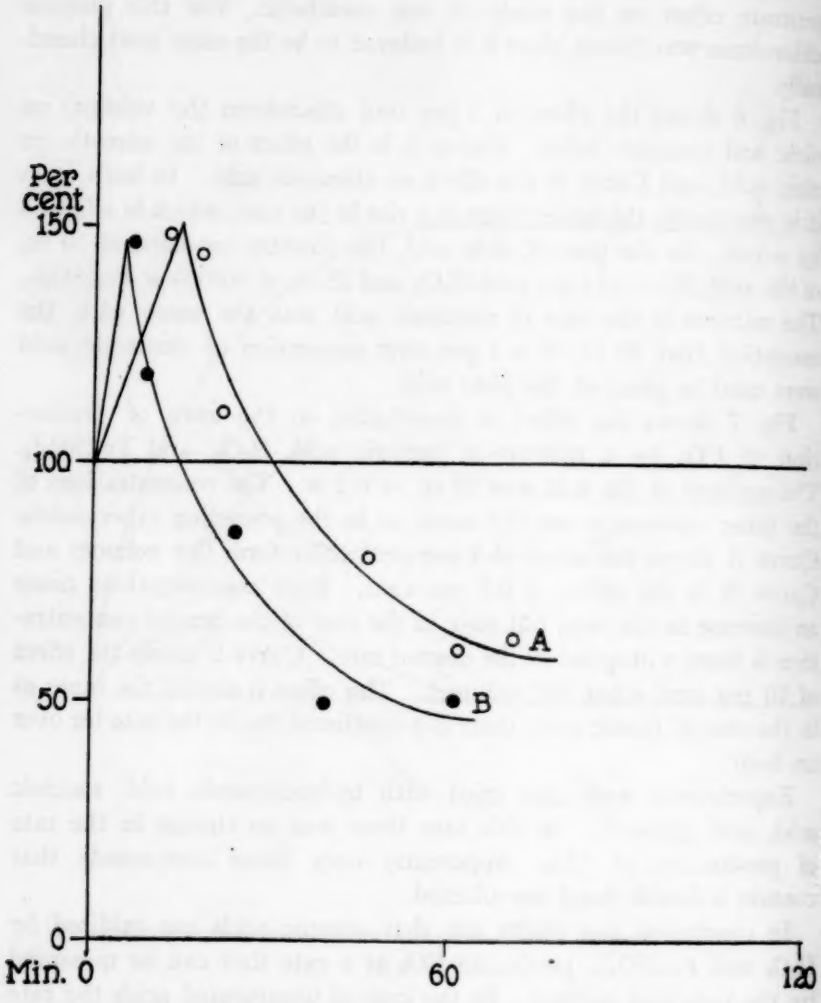

FIG. 6. The effect of the addition of 1 per cent chloroform on the rate of production of CO_2 by oleic acid (Curve A) and cinnamic acid (Curve B) in the presence of H_2O_2 and $Fe_2(SO_4)_3$. Each curve is the average of five experiments. The probable error of the mean is less than 10 per cent of the mean.

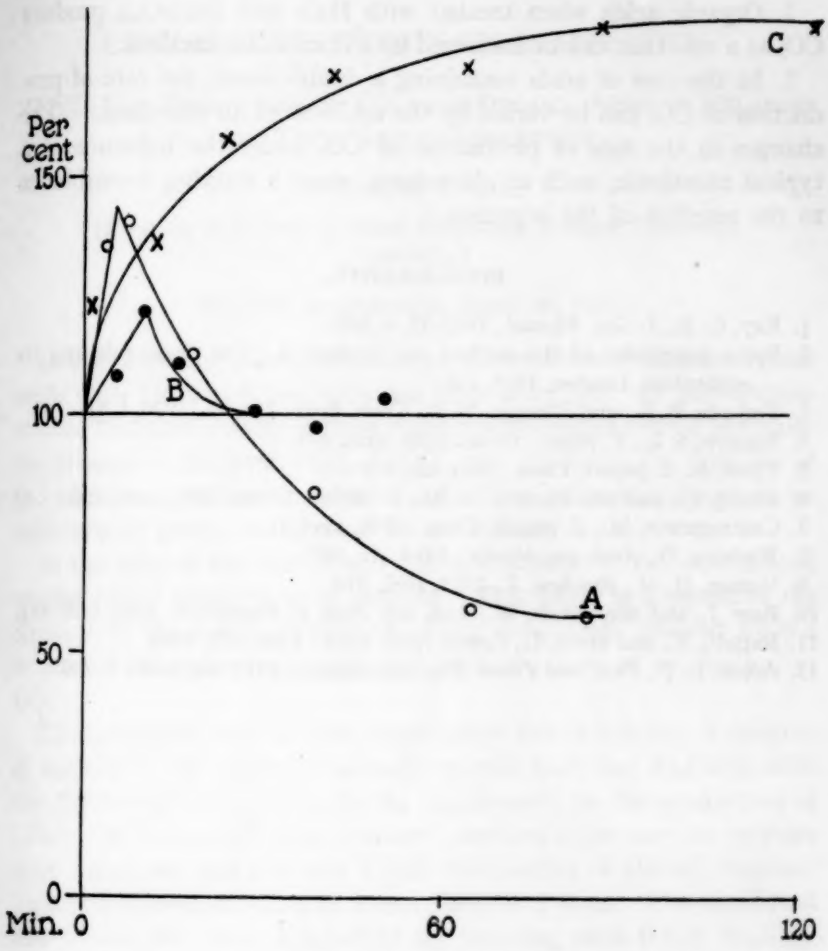

FIG. 7. The effect of anesthetics on the rate of production of CO_2 by fumaric acid in the presence of H_2O_2 and $Fe_2(SO_4)_3$. Curve A represents the addition of 1 cc. chloroform; Curve B the addition of 0.5 cc. Curve C is the effect of the addition of 10 cc. ether. Each curve is the average of five experiments. The probable error of the mean is less than 10 per cent of the mean.

SUMMARY.

1. Organic acids when treated with H_2O_2 and $Fe_2(SO_4)_3$ produce CO_2 at a rate that can be measured by the indicator method.

2. In the case of acids containing a double bond, the rate of production of CO_2 can be varied by the addition of an anesthetic. The changes in the rate of production of CO_2 under the influence of a typical anesthetic, such as chloroform, show a striking resemblance to the reaction of the organism.

BIBLIOGRAPHY.

1. Ray, G. B., *J. Gen. Physiol.*, 1922–23, v, 469.
2. For a description of the method see Findlay, A., The phase rule and its application, London, 1915, 246.
3. Verkade, P. E., and Söhngen, N. L., *Centr. Bakt., 2te Abt.*, 1920, l, 81.
4. Bigelow, S. L., *Z. physik. Chem.*, 1898, xxvi, 493.
5. Titoff, A., *Z. physik. Chem.*, 1903, xlv, 641.
6. Bredig, G., and von Berneck, R. M., *Z. physik. Chem.*, 1899, xxxi, 258.
7. Centnerszwer, M., *Z. physik. Chem.*, 1898, xxvi, 1.
8. Warburg, O., *Arch. ges. physiol.*, 1914, clv, 547.
9. Vernon, H. M., *Biochem. Z.*, 1912, xlvii, 374.
10. Baer, J., and Meyerstein, W., *Arch. exp. Path. u. Pharmakol.*, 1910, lxiii, 441.
11. Battelli, F., and Stern, L., *Compt. rend. Acad.*, 1905, cxli, 1044.
12. Acton, L. T., *Proc. and Trans. Roy. Soc. Canada*, 1913, vii, series 3, 209.

COMPARATIVE STUDIES ON RESPIRATION.

XXVI. The Production of CO_2 from Organic Acids in Relation to Their Iodine Absorption.

By GEORGE B. RAY.

(From the Laboratory of Plant Physiology, Harvard University, Cambridge.)

(Received for publication, March 20, 1923.)

In the preceding paper of this series[1] the reaction of various organic acids with H_2O_2 and $Fe_2(SO_4)_3$ has been described. It was demonstrated that the behavior of these compounds towards certain anesthetics, as regards the production of CO_2, was similar to the response of the organism. It was found that only those acids which contain an unsaturated group are affected by chloroform.

In the light of this fact it was felt that a study of the unsaturated groups might prove to be of interest. This paper is a report of the changes in the iodine absorption of the compounds affected by the action of chloroform, as compared with the rate of production of CO_2.

The technique used in these experiments was as follows: A mixture of the acid to be studied was made up with H_2O_2 and $Fe_2(SO_4)_3$ with the same concentrations as in the experiments on the production of CO_2[1]. At definite intervals measured portions of the reaction mixture were taken out and run into a definite quantity of Hanus' reagent.[2] This was allowed to stand in a cool place for 1 hour. The amount of free iodine was then determined by titrating with 0.1 N $Na_2S_2O_3$. Starch paste was used as an indicator. The results are represented in terms of the thiosulfate used in titrating the iodine.

[1] Ray, G. B., *J. Gen. Physiol.*, 1922–23, v, 611.

[2] This reagent consists of a solution of iodine monobromide and is made in the following manner. 13 gm. of iodine are dissolved in 1 liter of glacial acetic acid by means of a gentle heat, and to this solution 3 cc. of bromine are added. In these experiments the bromine is calculated as iodine.

The first experiments were made with oleic acid. Preliminary trials were made to determine the changes in iodine absorption due to the addition of H_2O_2 and $Fe_2(SO_4)_3$. In this case the test sample was dissolved in chloroform before the addition of Hanus' reagent. The results are shown in Fig. 1. (For convenience in this and in all the subsequent figures the curve of the rate of production of CO_2 is

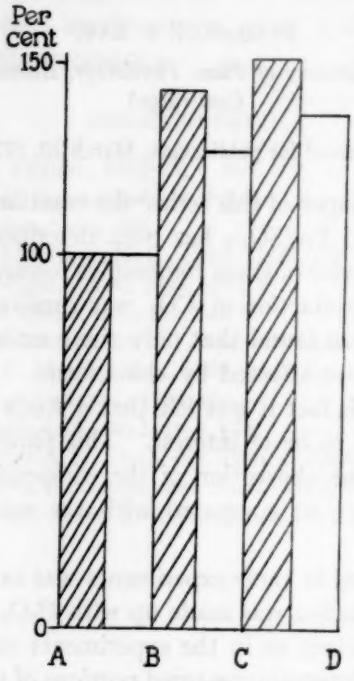

FIG. 1. The relation of the iodine absorption of oleic acid and the rate of production of CO_2. The shaded sections represent the CO_2, the unshaded the absorption of iodine. Section AB is oleic acid alone, Section BC oleic acid plus H_2O_2, and Section CD oleic acid plus H_2O_2 plus $Fe_2(SO_4)_3$.

given for comparison.) Section AB shows the amount of iodine absorption of the oleic acid alone; for convenience this is called 100 per cent. The shaded part represents the rate of production of CO_2. Portion BC is the iodine absorption when H_2O_2 has been added in the proportion of 25 cc. of 3 per cent solution to 50 cc. of oleic acid. It will be noted that there is an increase in the amount of iodine

absorbed and in the rate of production of CO_2. Section CD shows the effect of adding $Fe_2(SO_4)_3$ (25 cc. of 0.0004 solution to the mixture given above). In this case there is an increase in both curves. Control experiments were carried out to ascertain the influence of the peroxide and the iron on the iodine. They were found to be without effect.

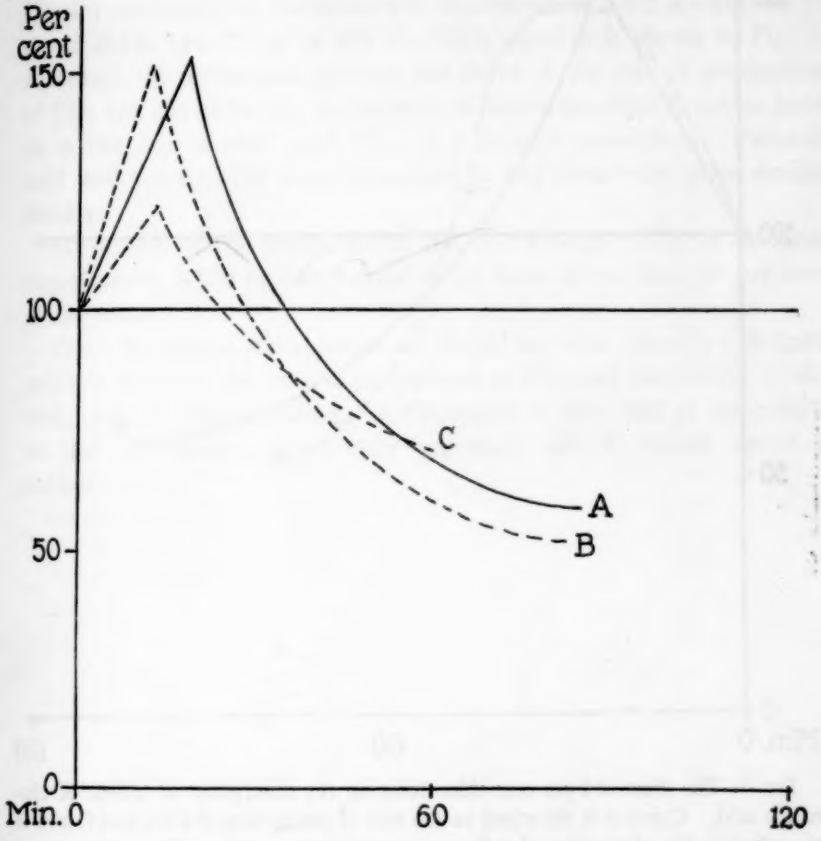

FIG. 2. The effect of chloroform on the absorption of iodine of oleic acid that has been treated with H_2O_2 and $Fe_2(SO_4)_3$. Curve A is the effect on the rate of production of CO_2, Curve B the effect of 1 per cent chloroform on the absorption of iodine, and Curve C the effect of 0.5 per cent chloroform on the absorption of iodine.

When chloroform was added to a mixture of oleic acid, H_2O_2, and $Fe_2(SO_4)_3$, in the proportions mentioned above, the iodine absorption underwent a peculiar change. If the results of these titrations are plotted against time it is found that the curve approximates the

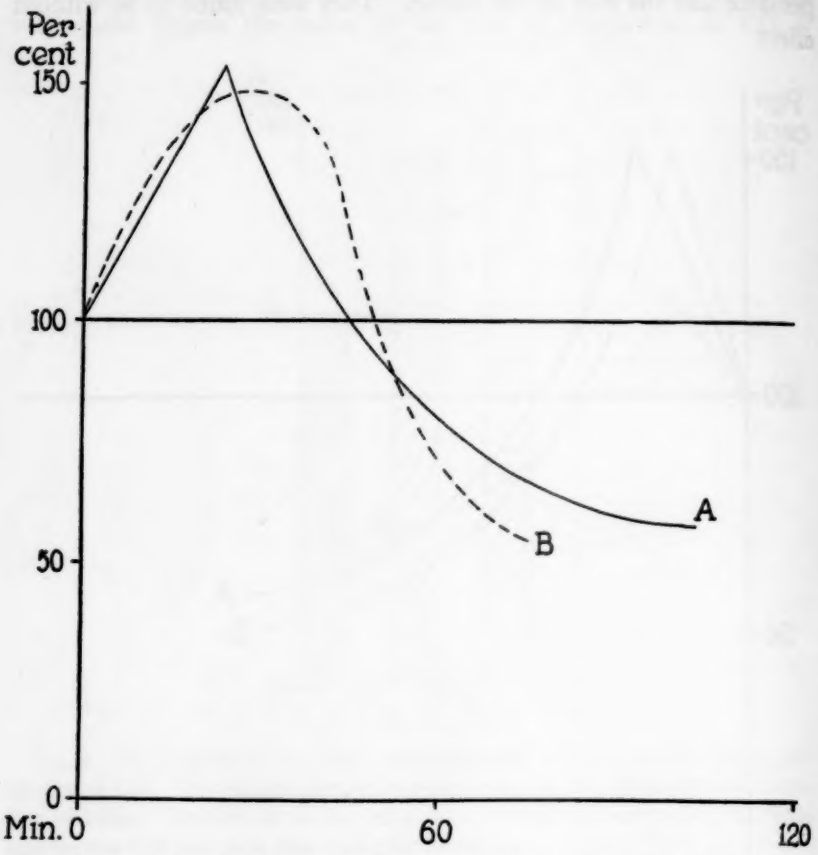

FIG. 3. The effect of 1 per cent chloroform on the absorption of iodine of cinnamic acid. Curve A is the effect on the rate of production of CO_2, and Curve B the effect on the absorption of iodine.

curve for the rate of production of CO_2. In Fig. 2 Curve A shows the effect of the addition of 1 per cent chloroform on the rate of production of CO_2 by 50 cc. of oleic acid in the presence of 25 cc. of the peroxide solution and 25 cc. of the iron, and Curve B illustrates the changes

in the iodine absorption of a similar mixture under similar conditions. The iodine absorption of the system in the absence of chloroform is called 100 per cent. That the chloroform is the factor that determines the reaction is shown by the action of 0.5 per cent chloroform (Curve C). Here we find that the reaction is less than when 1 per cent is used.

The addition of 1 per cent chloroform to a mixture consisting of 50 cc. of a suspension of cinnamic acid equivalent to a 0.2 M solution, 25 cc. of H_2O_2, and 25 cc. of the $Fe_2(SO_4)_3$ solution is shown in Fig. 3. Although the agreement between the curve of the rate of production of CO_2 and the curve for the amount of iodine absorbed is not as good as in the case of oleic acid, there is a definite parallelism. Fumaric acid and tannic acid when examined in the same way gave similar results.

All of the curves shown above are the average of three or more experiments. The probable error of the mean is less than 10 per cent of the mean.

From the above experiments one would say that there is a definite relation between the rate of production of CO_2 and the ability of the compound to absorb iodine. A discussion of this, and of the results of the experiments previously reported, will be taken up in a future paper.

THE COMPOSITION OF THE CELL SAP OF THE PLANT IN RELATION TO THE ABSORPTION OF IONS.

By D. R. HOAGLAND and A. R. DAVIS.

With the Assistance of J. C. Martin.

(From the Division of Plant Nutrition, College of Agriculture, University of California, Berkeley.)

(Received for publication, February 4, 1923.)

For a number of years, investigations have been carried on in this laboratory with the purpose of obtaining more accurate data than are now available concerning the absorption of ions by different plants under carefully controlled conditions. It is hoped that studies of this type may lead to a better understanding of the absorption processes as influenced by hydrogen ion concentration, by different concentrations of nutrient and of sodium salts, as well as by the environmental conditions of light and temperature. As part of these experiments, examinations have been made of the juices expressed from plants grown in definite culture solutions. It has been frequently pointed out, however, by Osterhout[1] and others that the juices expressed from complex plant tissues do not represent an unaltered cell sap, because in crushing the cells, the sap may be changed as a result of adsorption, or chemical reaction, or because of admixture with intercellular substances. Moreover, the pH of such sap may be greatly influenced by the liberation of materials stored up in dead or special cells, as is the case in various citrus-fruits.

The observations of Wodehouse[2] and of Crozier[3] on *Valonia* directed the attention of the writers to the possibility of extending the scope of our information through the study of the vacuolar sap obtained from individual plant cells. At the suggestion of Dr. N. Gardner of the Department of Botany, we have used the fresh water alga *Nitella*

[1] Osterhout, W. J. V., *J. Gen. Physiol.*, 1922–23, v, 225.
[2] Wodehouse, R. P., *J. Biol. Chem.*, 1917, xxix, 453.
[3] Crozier, W. J., *J. Gen. Physiol.*, 1918–19, i, 581.

(*Nitella clavata*) for this purpose. Meanwhile, Osterhout,[4] Brooks,[5] and Irwin[6] have published several very interesting and suggestive papers dealing with experiments in which an eastern species of *Nitella* has been employed. Since the questions involved are of outstanding importance to the investigation of several aspects of plant nutrition, it is possible that the additional discussion and data now presented from a somewhat different point of view may not be without interest. As will be indicated later, some of the results have an obvious bearing on certain current theories relating to the intake of ions by the higher plants. This paper is also primarily concerned with the lower salt concentrations, such as are found in culture and soil solutions favorable to the growth of common plants.

The *Nitella* cells used in these experiments were collected from a small pond. Most of the cells were quite small, but many could be found which were from one to several inches in length. The cells were very thin walled and turgid, and it was discovered that they could be easily broken in the middle, with the rather forcible ejection of a small drop of sap. By working with a large number of cells, a quantity of sap could be secured adequate for chemical analysis by suitable special methods. This sap was almost clear, except for a few chloroplasts which rapidly settled to the bottom of the test-tube. In most instances, the sap was filtered and diluted before proceeding with the chemical determinations. Except where otherwise indicated, the cells were kept in tap water. The cells were always rinsed thoroughly with distilled water before making tests or analyses, except in the first experiment. The methods of analysis were essentially those described by Stewart[7] as adapted to the examination of soil extracts. Even with the comparatively small volumes of sap available (from 3 to 15 cc. in different experiments), the accuracy of the analytical technique is quite sufficient to justify the type of conclusions which are drawn in this paper.

The sap for the first experiment to be described was collected from the largest cells available (from 1 to 3 inches in length), immediately

[4] Osterhout, W. J. V., *J. Gen. Physiol.*, 1921–22, iv, 275.
[5] Brooks, M. M., *J. Gen. Physiol.*, 1921–22, iv, 347.
[6] Irwin, M., *J. Gen. Physiol.*, 1922–23, v, 223.
[7] Stewart, G. R., *J. Agric. Research*, 1918, xii, 311.

after their removal from the pond water. Only healthy cells were included, as far as could be judged from turgidity and general appearance. A sample of the water in which the plants were growing was also taken at the same time, and this was analyzed by procedures comparable to those adopted for the sap. The results of the analyses, as well as pH values, resistances, and freezing point depressions are cited in Table I.

Clearly every element was present in the sap in very much higher concentration than in the surrounding medium, but the factors of concentration are strikingly different for the different elements. Potassium, chlorine, and sodium have higher factors than sulfate, calcium, or magnesium. The concentration of potassium actually dissolved in the pond water must have been exceedingly small (no weighable precipitate with platinum chloride was obtained from a 200 cc. sample), but replacement from suspended colloidal material could take place as fast as absorption by the plant proceeded. The sodium determination is, of course, less accurate than the others, and subsequent analyses gave higher values. Variations also occurred with calcium and magnesium. These are explained possibly by the variations of sap composition with cells of different sizes, and by the different solutions in which the cells were kept at the laboratory. It should be noted, however, that the general relations of the ions and the magnitudes of concentration for potassium and chlorine were quite similar, except where the cells were placed in special solutions for studies on absorption, to be referred to presently.

Several points of difference are found between the composition of *Valonia* sap as reported by Osterhout,[1] and that of *Nitella*. In the latter, no appreciable quantity of nitrate was found (although 34 parts per million were present in the pond water), while the *Valonia* sap contained more nitrate than the sea water. Practically no sulfate was found in *Valonia*, but very definite quantities were found in the *Nitella* sap. Magnesium was found in *Valonia* only in traces. The conductivity of the *Nitella* sap was twenty-five times as great as that of the surrounding water, which is a far greater difference than that existing between *Valonia* sap and sea water. This comparison is in agreement with Osterhout's observations on the conductivity of *Nitella* sap.

TABLE I.

Analyses of Nitella Sap and of Pond Water.

	Specific resistance (25°C.).	Freezing point depression.	pH	K		Na		Ca		Mg		Cl		SO_4		PO_4		NO_3	
				P.P.M.	Milli-equivalent.	P.P.M.	Milli-equivalent.	P.P.M.	Milli-equivalent.	P.P.M.	Milli-equivalent.	P.P.M.	Milli-equivalent.	P.P.M.	Milli-equivalent.	P.P.M.	Milli-equivalent.	P.P.M.	Milli-equivalent.
	ohms																		
Sap from largest cells (from pond).....	82.3	0.465°C.	5.2	2,120	54.3	230	10.0	410	20.5	430	35.4	3,220	90.7	800	16.7	350	3.7	0	0†
Pond water.....	2,050	0.018°C.	7.2–9.4	*	—	5	0.2	31	1.6	41	3.4	32	0.9	31	0.7	0.4	0.004	34	0.5
Factor of concentration.....	25	26	—	—	—	46		13		10		100		26		870			
Other analyses (partial).																			
Small cells (3 days in culture solution).....				1,780	45.7	1,350	58.8	900	45.0	710	59.2	—	—	1,100	22.9	250	2.6		
Large cells (from tap water)..				1,810	46.5	—	—	800	40.0										
" " (" " ")..				1,750	44.8	870	37.8												
" " (" peat extract).				2,200	56.4	—	—	600	30.0	430	35.8	3,200	90.1	—	—				
" " (" tap water).												3,500	98.5			390	4.0		

* No weighable precipitate of potassium chloroplatinate from 200 cc. of water.

† No test with diphenylamine.

That the major portion of the inorganic elements of *Nitella* sap exists in ionic state, seems to be a necessary conclusion from the measurements of conductivity, which showed that the sap had a conductivity slightly greater than that of an 0.1 N KCl solution, as well as from other considerations presented below. Moreover, if the positive and negative ion equivalents are compared, it is possible to balance 80 to 90 per cent of the positive ions by negative ions as determined. The remaining equivalents of negative ions may very plausibly be ascribed to organic acid anions.

With regard to the phosphorus, the quantity obtained by direct precipitation with the phosphomolybdate reagent was as great as that found when the sap was evaporated and the residue ignited with MgO. The sap yielded a precipitate of $BaSO_4$ directly on the addition of $BaCl_2$. The sap from individual cells of normal appearance also gave microchemical tests for sulfate. These observations suggest that the phosphorus and sulfur were present in ionic state, and that the presence of sulfate in *Nitella* sap is not an indication of injury, as seems to be the case with *Valonia*.

An artificial solution was then prepared to imitate the inorganic content of the sap, except for the undetermined organic anions and the equivalent positive ions. The conductivity of this solution was compared with that of the sap with the following results.

Salt mixture, specific resistance, 97.7 ohms
Cell sap " " 82.3 "

The two values are evidently very similar, but the sap possesses an approximately 20 per cent greater conductivity than the salt mixture, the discrepancy being attributable mainly to the undetermined ions omitted from the artificial solution.

The freezing point depression of the sap was 0.465°C., and that of a KCl solution of the same conductivity, 0.395°C., or 85 per cent of the total.

The total solids were determined and the organic matter estimated by just charring the residue, extracting with water, and then heating the insoluble part very carefully at a low temperature to drive off the carbon. The total volatile matter was less than 25 per cent of the total solids. The total nitrogen of the cell sap was determined and

found to be only 160 parts per million. Nitrogen partitions on expressed sap indicated that amides, amino-acids, and very small proportions of ammonia were in solution in the sap.

From these various considerations, it is reasonable to conclude that no important portion of the inorganic elements contained in the sap is held in organic combination or in non-dissociated form. A similar conclusion has been stated very recently by Osterhout with reference to potassium and certain other inorganic elements of *Valonia* sap.

Some interest may also attach to the determinations of the inorganic elements held in insoluble form in the cell wall or protoplasm. A mass of cells of healthy appearance (previously placed in peat extract to remove calcium salts adhering to the surfaces of the cells) was sub-

TABLE II.

Inorganic Elements of Nitella Cells Insoluble in Water.

	Dry material, per cent
Ca	5.40
Mg	0.19
K	0
Al	0.15
Fe	0.06
PO_4	1.40
SiO_2	1.30
SO_4	1.09

jected to the pressure of a screw-press, and the sap removed as thoroughly as possible. The residue was then washed many times with distilled water, and was also subjected to the action of a direct electric current. After removal of all excess water, the moist cake was dried and ashed at a low temperature. This ash was then analyzed, the data so obtained being shown in Table II. It is perhaps of some significance that calcium is the chief constituent of the ash. Probably most of the calcium, magnesium, and sulfur existed in the cell residue in organic combination, but no potassium could be detected. No examination was made for sodium. Appreciable quantities of silica were found in the residue, but none in the sap. Iron and aluminum were both present in the cell residue in appreciable quantities.

Since the sap from the large *Nitella* cells may be obtained practically uncontaminated, an opportunity is offered for comparing the cell sap with the juices expressed from the whole mass of cells. To accomplish this comparison, large cells were selected, all from the same jar of tap water. From one portion, the sap was collected from individual cells in the manner already described, while the other portion, made up of cells of sizes similar to those of the first portion, was pressed as thoroughly as feasible with a screw-press. This expressed juice was filtered clear, and both the cell sap and the expressed sap were examined for conductivity and for Ca, Mg, K, and Cl (Table III). The conductivity measurements showed that the expressed sap was

TABLE III.

Comparison of Sap from Individual Cells and Expressed Sap.

	Specific resistance.	K P.P.M.	Cl P.P.M.	Ca P.P.M.	Mg P.P.M.
	ohms				
Cell sap.	81	2,200	3,200	600	430
Expressed sap.	120	1,310	2,250	310	150
Dilution factor.	1.48	1.68	1.43	1.94	2.82

1.5 times more dilute than the unchanged cell sap. The concentrations of chlorine and potassium were almost in the same proportion as the conductivities. In other words, the sap was diluted to that extent with regard to these elements, this dilution being caused, in large part, no doubt, by the water adhering to the outside of the cells which it was not feasible to remove completely. Magnesium, however, is found to be in distinctly less concentration in the expressed sap than would be indicated by the relative conductivities. To a less degree, possibly the same is true of calcium. Evidently the magnesium adsorbed can be removed by sufficiently drastic washing, as already noted. It may be added that it is quite possible that preliminary freezing would have lessened the adsorption of magnesium and calcium.

Hydrogen Ion Concentration of Sap.

The hydrogen ion concentration of the sap was determined by the indicator method and by the hydrogen electrode. In a comparative

test of one sample of sap, the former method gave a value of pH 5.2, the latter pH 5.1. A large number of individual cells were also examined by a spot plate method. Out of several hundred apparently normal cells tested, less than a dozen varied in their reaction from pH 5.2 within the experimental error (from 0.1 to 0.2 pH). The extreme limits were 4.8 to 5.8. The pH values of different *Valonia* cells as reported by Crozier[3] showed less constancy.

In order to determine whether the hydrogen ion concentration of the sap could be changed easily, cells were placed in culture solutions adjusted to different pH values, and after a period of several days, tests were made of the reaction of the sap from numerous cells removed from the different solutions. The results are noted in Table IV.

TABLE IV.

Hydrogen Ion Concentration of Sap and Culture Solutions.

Solution.	pH of solution.	pH of sap.	Remarks.
Culture.*	3.8	3.7	Decided injury.
"	4.4	4.7	Slight "
"	5.0	5.0	No "
"	5.6	5.2	" "
"	7.0	5.1	" "
Tap water.	7.2–9.4	5.2	" "
Peat extract.	6.8	5.0–5.2	" "

* General culture solution used for growing plants, total concentration about 1,500 P.P.M., hydrogen ion concentration adjusted with NaOH and HCl.

On the alkaline side, the plants, during the active period of photosynthesis, changed the reaction of the tap water to pH 9.4 or greater. Thus, the total range of hydrogen ion concentration of the media included in the observations was from pH 3.8 to 9.4+, yet under the conditions of the experiment, no appreciable change of pH occurred in the cell sap, except below pH 5, and at the lower values the cells were unquestionably injured. In general, it was found that any appreciable change of reaction in the cell was accompanied by injury. In such cases, some chlorine always diffused out of the cell. In fact, this exosmosis of chlorine seemed to be a delicate test for injury or altered permeability, since excretion of chlorine could often be demonstrated some time before there was any other visible indication of abnormality. The maintenance of an approximately constant

hydrogen ion concentration in plant cells of this type may reasonably be supposed to be of great significance to normal functioning, in view of Loeb's findings on the importance of the relations between proteins and the pH values of the solution. As already stated, Crozier found the reaction of the sap from different *Valonia* cells less constant than that of *Nitella*, as observed by the writers; but he concludes that the reaction of the *Valonia* cells bears no definite relation to the reaction of the outside medium.

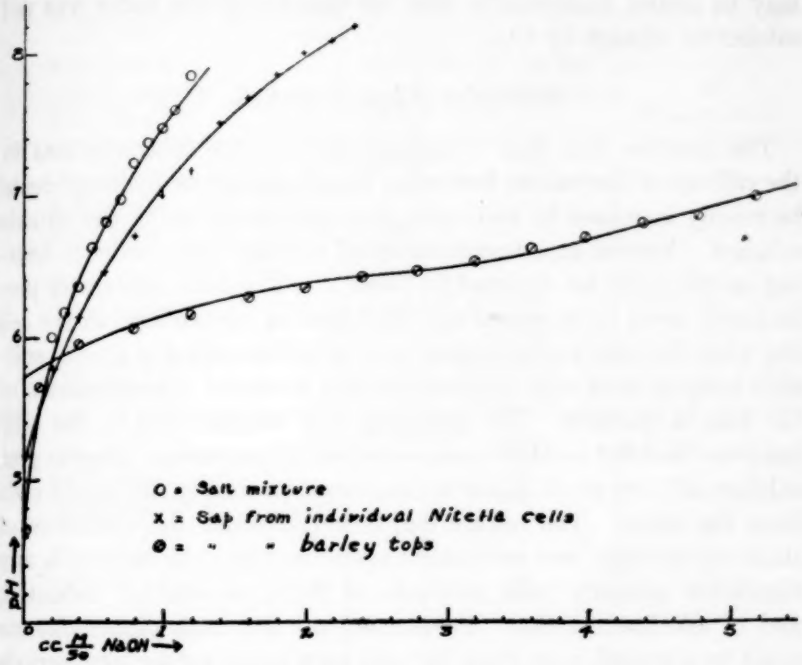

FIG. 1. Titration curves for *Nitella* sap. Artificial salt mixture and barley juices.

The juices expressed from plant tissue have been shown by Haas,[8] Hempel,[9] and others to possess a considerable buffer effect. It seemed probable that the *Nitella* sap also was buffered and a titration was carried out in the customary manner with the aid of a hydrogen electrode (Fig. 1). In addition, a similar titration was made on the

[8] Haas, A. R. C., *Soil Sc.*, 1920, ix, 341.

[9] Hempel, J., *Compt. rend. trav. Lab. Carlsberg*, 1917, xiii, 130.

salt solution partially imitating the cell sap. For comparison, a titration of the juices expressed from actively growing barley plants is also included. The buffer effect of the *Nitella* sap is very appreciable and greater than that of the salt mixture from which was omitted a certain proportion of cations in equilibrium with unknown organic anions. These latter compounds must also have contributed to the buffer system, as well as the phosphates. The titration value of the expressed barley juices greatly exceeded that of the *Nitella* sap. It may be stated, incidentally, that the reaction of the latter was not subject to change by CO_2.

Absorption of Ions by the Cell.

The question was then considered whether the concentrations in the cell sap of the various ions other than hydrogen or hydroxyl could be readily increased by increasing their concentrations in the outside solution. Several experiments designed to yield some evidence bearing on this point are reported in Table V. The data, while only preliminary, point to increased concentrations of certain ions in the cell sap when the cells are in contact for a sufficient period of time at suitable temperatures with solutions having increased concentrations of the ions in question. The potassium and chlorine ions in the cells immersed in 0.005 and 0.01 molar solutions of potassium chloride plus calcium salt, are much higher in concentration than in any of the cells from tap water. The nitrate ion, however, under the conditions of these experiments, was not found to penetrate into the cell sap in any significant quantity with solutions of the concentration indicated, and of alkaline reaction. Frequently, no test with diphenylamine could be detected, even when the cells were immersed for long periods in nitrate containing (0.001 M to 0.005 M) solutions. The test,when present, was slight. Control tests showed that very minute quantities of nitrate added to the sap gave a deep color with the reagent.

In certain experiments conducted by Theron[10] and by the writers on the absorption of NO_3 ion by higher plants, it has been determined that, all other conditions being equal, the removal of NO_3 is significantly more rapid from slightly acid solutions than from slightly

[10] Theron, J. J., *Univ. California Pub. Agric. Sc.*, 1923, (in press).

alkaline solutions, and was accompanied by superior plant growth in the former case. We were, therefore, desirous of taking advantage of the opportunity afforded by the use of *Nitella* to examine directly

TABLE V.

Concentrations in Cell Sap from Cells in Various Solutions.

Solution.	K P.P.M.	Cl P.P.M.	NO_3	Time of exposure.
Experiment 1.				
				days
KNO_3, 0.01 M $Ca(NO_3)_2$, 0.005 M			No test by phenoldisulfonic reagent.	10
KCl, 0.01 M	‡	8,300		Warm weather.
Experiment 2.				
KCl, 0.001 M $CaCl_2$, 0.001 M	1,970	3,270		
KCl, 0.005 M $CaCl_2$, 0.005 M	3,500	4,720		28
KCl, 0.01 M $CaCl_2$, 0.01 M	‡	4,940		
Experiment 3.				
KNO_3, 0.004 M $Ca(NO_3)_2$, 0.004 M	1,740		*	
KNO_3, 0.012 M $Ca(NO_3)_2$, 0.012 M	1,760		*	18.
KNO_3, 0.016 M $Ca(NO_3)_2$, 0.016 M	1,710			
Tap water.	1,820	3,500	†	

* Test with diphenylamine, but NO_3 present was too small in amount for satisfactory quantitative determination.

† No test.

‡ Insufficient sample for determination.

the penetration of nitrate ion into the cell sap, when the cells were immersed in solutions of different hydrogen ion concentrations. Two separate experiments were made and the results were quite definite

in showing that the reaction of the media had a determining influence on the absorption of nitrate into the cell sap. The solutions were buffered with phosphate and contained 0.005 M NO_3 as KNO_3. The penetration of nitrate occurred readily at pH 5.0 to 6.0, less readily at pH 7.2; and at pH 8.5, practically no penetration occurred during the period of the experiment. This conclusion was reached both from tests on the sap with diphenylamine reagent and from quantita-

TABLE VI.

Influence of Hydrogen Ion Concentration on Penetration of NO_3 into Nitella Sap.

Experiment 1.

Solutions buffered with phosphates, 0.002 M, and KNO_3, 0.005 M added.

Solution.	pH of solution at beginning.	pH of solution at end.	NO_3 tests on sap with diphenylamine.
A.	5.2	5.9	Heavy test in all cells at end of week.
B.	7.0	6.8	Slight test in nearly all cells at end of week.
C.	9.0+	7.4	Practically all cells free of test at end of week.

Experiment 2.

Solutions buffered with phosphates, 0.01 M, and KNO_3, 0.005 M added.

Solution.	pH of solution at beginning.	pH of solution at end.	NO_3 in sap,* P.P.M.	NO_3 tests on sap with diphenylamine.
A.	5.0	5.9	4.2	Heavy test in all cells at end of 12 days.
B.	6.2	6.4	1.6	Distinct test in all cells at end of 12 days.
C.	7.2	7.2	0.9	Very slight test in practically all cells at end of 12 days.
D.	8.5	8.3	0	Practically all cells free of test at end of 12 days.

* By phenoldisulfonic acid method. In each solution about 50 separate cells were examined; solutions kept at temperature of room (15–20°C.).

tive estimations made by the phenoldisulfonic acid method, alumina cream being used as a clarifying agent (Table VI). Irwin[6] has reported in a note recently published that the penetration of the dye, brilliant cresyl blue, is very greatly affected by the hydrogen ion concentration of the solution.

In connection with the absorption of nitrate, it may be worth while to state that a deep blue color was nearly always developed when the whole cell was dipped in the diphenylamine reagent, even when the

cell sap gave no test. It is possible that nitrate, combined in some way in the cell wall or protoplasm, is freed by the sulfuric acid of the reagent, although it is difficult to prove that some other oxidizing substance might not give a similar test. A careful analysis was made up of a sample of expressed sap by the zinc-copper reduction method for nitrate, blank determinations being made at the same time. Very closely agreeing duplicate analyses indicated that small amounts of nitrate were present in the expressed juice (an average of 5.9 parts per million nitrate nitrogen). It would seem that the possibility exists that nitrate may form some sort of initial combination in the cell wall or protoplasm, and that this combination and also the penetration of NO_3 into the cell sap are influenced by the hydrogen ion concentration of the solution. It may be emphasized here that the solutions employed in these experiments were much more dilute than those generally used in studies on antagonism.

Numerous studies have been made from time to time in this laboratory on the effect of the plant (barley, peas, cucumbers), on the reaction of the solutions in which the roots are immersed. With several salts, marked changes of reaction are brought about by seedlings. Ammonium salts are especially susceptible to such alteration of reaction, the solution becoming more acid to the extent of approximately pH 3.2. We are indebted to the work of Jacobs[11] for direct experiments on the mechanism of changes of reaction analogous to those just mentioned. This investigator has shown that ammonium penetrates into the living cell very rapidly, bringing about an increased alkalinity, even when the outside solution is acid.

It was evident that some interest would attach to observations on the effect of ammonium salts on the reaction of *Nitella* sap. A number of tests were carried out with ammonium sulfate, chloride, and nitrate in low concentrations. As was expected, the ammonium ion penetrated rapidly and caused a change of reaction in the cell sap. From 0.005 M ammonium salts, the reaction was, in most cases, changed from pH 5.2 to pH 5.6 to 6.2 in the course of 24 hours or less. The cells were injured and exosmosis of chlorine quickly occurred. It happened in one instance that certain cells, immersed for about 12 hours in a 0.005 M solution of ammonum sulfate, showed no exos-

[11] Jacobs, M. H., *J. Gen. Physiol.*, 1922–23, v, 181.

mosis of chlorine, although the reaction of the sap had been changed, but a few hours later chlorine was found in the solution. The experiments so far made seem to point to a change in permeability with a change of reaction of the sap, very soon followed by outward diffusion of chlorine. The exosmosis of other ions may be much less rapid. For example, when ammonium nitrate was used, chlorine was found in the solution before any sulfate or phosphate had diffused out.

With solutions as dilute as 0.005 M, the effect of the ammonium salt may be indefinitely delayed with some cells, apparently more resistant than others. Temperature is also very important. Thus in one experiment with 0.005 M $(NH_4)_2SO_4$, exosmosis of chlorine was detected within 12 hours when the temperature of the solution was maintained at 25°C., while at 10°C., it required several days for the effect to become apparent. The presence or absence of calcium salts in 0.005 molar concentration did not seem to have any easily recognizable influence on the penetration of ammonium from these dilute solutions. Subsequent and more extensive experiments have shown that the amount of Cl diffusing out of the cells may be lessened even by this concentration of calcium.

Investigations on the higher plants prove that the absorption of nitrate almost always proceeds at a more rapid rate than that of any cation, equilibrium in the solution being maintained by the formation of HCO_3 ion. In order to determine whether an analogous effect could be demonstrated with *Nitella*, cells were placed in solutions of KNO_3, 0.005 M, and KNO_3, 0.005 M, plus $Ca(NO_3)_2$, 0.005 M, and the changes of reaction of the solutions were noted. The cells had previously been in contact with peat water for a long period, and no surface deposits were present. After several weeks, the reactions of the solutions, as determined during the period of photosynthesis, were found to be above pH 9.0, the original reactions being at pH 5.0. In order for this change to occur, NO_3 must have been absorbed to a greater extent than the cation with the formation of bicarbonate, and subsequently a high alkalinity developed, conditioned, of course, by the photosynthetic utilization of carbon dioxide.

The question arose as to the extent to which a solution could be freed of chlorine when in contact with *Nitella* cells. A simple experiment was carried out by placing a large number of cells in a solution

containing 15 parts per million KCl. After several weeks at room temperature, every trace of chlorine had disappeared from the solution, as shown by tests with silver nitrate. In a comparable test with a dilute solution of potassium nitrate, the nitrate was also completely removed.

Since the different ions are retained in the cell sap at concentrations very much higher than in the surrounding solution, it may be asked whether outward diffusion can take place with normal cells. For this purpose, Cl may be tested for very conveniently. A large number of cells were very carefully washed and then placed in distilled water. No test for Cl was given after 16 days. A faint test present at 9 days disappeared later. No doubt a few injured cells had given up their chlorine, but this was quickly absorbed by the healthy cells. It was thought that the normal permeability might have been maintained in this case by reason of the calcium derived from surface precipitates which regularly accumulate on the cells when growing in tap water. This may be removed by allowing the cells to remain for some time in a very slightly acid extract of peat (which was found to be a very favorable medium). Cells freed in this way of surface deposits of calcium were also placed in distilled water. At the end of 3 days, a slight chlorine test was present, but later the solution was completely freed of chlorine and the cells appeared normal at the end of 3 weeks. Other cells were placed in a solution containing sodium in high concentration (0.1 N NaCl and 0.01 N $CaCl_2$). Tests were made on this solution for the exosmosis of potassium, but the cobalti-nitrite reagent failed to show any trace of this element during the period of 2 weeks. It would seem that these cells under normal conditions possess a unidirectional permeability with reference to such elements as potassium and chlorine.

It will now be desirable to make a very brief comparison of the *Nitella* cell sap and the juices expressed from more complex plants. It is highly essential to an understanding of the metabolism of such plants to gain some deeper insight into the forms in which the inorganic elements are held by the plant, and no doubt the experiments on *Nitella* may serve to a certain extent as a guide in the interpretation of the available data. For example, the conductivity of *Nitella* sap and of the juices from barley plants grown in a favorable medium of

comparable concentration is of similar magnitude, as is the general relation of the cations. The tissues of different plants have been examined at various stages of growth for water soluble inorganic elements, and we have found that in every case practically all of the potassium is easily soluble in water, as well as the major portion of the other elements, with a few exceptions in the case of calcium. This element is known to form insoluble combinations with organic acids in some plants. Referring to another case, experiments conducted a number of years ago in this laboratory[12] on certain of the giant kelps (*Macrocystis pyrifera*, and *Nereocystis luetkeana*) of the Pacific Coast, yielded evidence that the potassium and other inorganic elements were easily soluble in water and that the usual ionic reactions were given. The idea which had often been advanced, that in these plants potassium was held in some sort of organic or non-dissociated form, did not appear to be correct.

On the whole, the presumption is very strong that generally a large proportion of the inorganic elements found in most plants is not in organic combination, and is very likely in dissociated form. It is also at least suggestive that the greater selectivity for potassium, sodium, and chlorine, than for calcium, magnesium, and sulfate, displayed by *Nitella*, is paralleled by such plants as barley and wheat. The latter absorb chlorine, for example, very readily, if suitable concentrations are present in the culture medium. The idea which is frequently held that plants simply absorb the essential elements and reject the others has no basis in fact. With transpiring plants, many complications are, of course, introduced, but the essential principles of absorption by cells and those relating to the functions of the inorganic elements are probably not different from those applying to the simpler water plants.

The large buffer effect found in the juices of common plants is, no doubt, the result mainly of the relatively high concentration of organic acid anions in equilibrium with various cations, especially potassium. While *Nitella* sap also has a high content of potassium, this is balanced to a large extent by the anion chlorine, instead of by organic anions, and the buffer effect is correspondingly less than that of the higher plants. Considerable new growth of *Nitella* can be produced in a

[12] Hoagland, D. R., *J. Agric. Research*, 1915, iv, 39.

culture solution free of chlorine (except for such traces as are derived from a few dead cells), but it is questionable whether continued normal growth can occur in the absence of chlorine. It is difficult to see what anion could replace it when an ordinary culture solution is used.

Recently, a number of theories have been advanced to explain the "feeding powers" of plants. One of these states that some plants absorb potassium more readily than others from very dilute solutions, because of some sort of precipitation or adsorption of this element in the plant, which brings about a constant flow of potassium inward. It is considered that plants having juices with pH values relatively higher than those of the soil solution are, as a consequence, better able to effect a precipitation of potassium, and are, therefore, better "feeders" in this regard. *Nitella* offers a direct contradiction to this and similar theories. Absorption of potassium takes place from a very dilute solution of this element without precipitation in the plant, and, moreover, the cell sap has a decidedly more acid reaction than the water from which the plants absorb their inorganic elements.

The general question of selective action by plants as illustrated by *Valonia* is briefly discussed by Osterhout[1] in a recent article published in this *Journal*. The conclusion is reached that the selection of potassium by cells cannot always be explained on the basis of the formation of non-dissociated compounds. It is also evident that it will be necessary to explain the retention in the cell, not only of potassium, but also of chlorine and other ions, both positive and negative. There is, in addition, the difficulty that ions may be absorbed from a surrounding solution of low concentration into a solution of much higher concentration. The mechanism of these processes, in which energy relations may have to be taken into account, is not yet clear. The more definite understanding of selective action must await the outcome of such experiments as those being conducted by Loeb, Osterhout, and other workers in the field of general physiology. It is only by researches of this type that the nature of the chemical reactions fundamental to the study of plant nutrition can be elucidated.

SUMMARY.

1. Chemical examination of the cell sap of *Nitella* showed that the concentrations of all the principal inorganic elements, K, SO_4, Ca, Mg,

PO_4, Cl, and Na, were very much higher than in the water in which the plants were growing.

2. Conductivity measurements and other considerations lead to the conclusion that all or nearly all of the inorganic elements present in the cell sap exist in ionic state.

3. The insoluble or combined elements found in the cell wall or protoplasm included Ca, Mg, S, Si, Fe, and Al. No potassium was present in insoluble form. Calcium was predominant.

4. The hydrogen ion concentration of healthy cells was found to be approximately constant, at pH 5.2. This value was not changed even when the outside solution varied from pH 5.0 to 9.0.

5. The penetration of NO_3 ion into the cell sap from dilute solutions was definitely influenced by the hydrogen ion concentration of the solution. Penetration was much more rapid from a slightly acid solution than from an alkaline one. It is possible that the NO_3 forms a combination with some constituent of the cell wall or of the protoplasm.

6. The exosmosis of chlorine from *Nitella* cells was found to be a delicate test for injury or altered permeability.

7. Dilute solutions of ammonium salts caused the reaction of the cell sap to increase its pH value. This change was accompanied by injury and exosmosis of chlorine.

8. Apparently the penetration of ions into the cell may take place from a solution of low concentration into a solution of higher concentration.

9. Various comparisons with higher plants are drawn, with reference to buffer systems, solubility of potassium, removal of nitrate from solution, etc.

THE EFFECT OF RADIO-ACTIVE RADIATIONS AND X-RAYS ON ENZYMES.

I. The Effect of Radiations from Radium Emanation on Solutions of Trypsin.

By RAYMOND G. HUSSEY and WILLIAM R. THOMPSON.

(From the Laboratories of The Memorial Hospital and the Department of Pathology, Cornell University Medical College, New York.)

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At the present time our information concerning the changes effected in living matter by x-rays and the radiations from radio-active substances is confined chiefly to the characteristic exudative phenomena noted in animal tissues, and the altered structural character of cells observed following exposures to these radiations. We have but little data bearing on the underlying physiological processes which lead to these characteristic morphological changes. Several speculations have been made from time to time to explain the biological action of these radiations, but no experimental evidence has been presented for their support. It is obvious that an analysis of the physiological action of these physical agents depends upon data to be obtained from investigations which will throw some light on the chemical action of x-rays and radio-active radiations on living matter. Since at this time we have no direct experimental approach to cell chemistry, we have to content ourselves with inquiries concerning the effect of the radiations under discussion on types of biological reactions which can be studied *in vitro*. Since enzymes hold such a dominant place in the chemistry of living matter, the reactions of these substances appear to be the logical ones to investigate first. Moreover, for these investigations we have methods for observation which permit one to obtain results that can be treated quantitatively.

In this communication we will report the results of experiments in which we have observed the effect of radiations from radium emanation on aqueous solutions of powdered trypsin. The source of radiation

employed for the experiments to be reported in this paper was purified radium emanation contained in sealed capillary glass tubes of about 13 mm. length and 0.4 mm. outside diameter. The wall thickness of the tubes used (approximately 0.1 mm.) prohibits the passage of alpha radiation but interferes very little with the passage of the beta and gamma radiation. (The method of collecting emanation in this laboratory and of measuring its activity is fully described in a paper by Failla.[1] We are indebted to Mr. Lobley of our Radium Laboratory for the special preparation of the tubes for our experiments.)

In order to have uniform conditions of exposure, the following procedure was found to give satisfactory results. A disk was punched out of a block of paraffin with a cork borer and the emanation tube was set in its center by means of a short extension of glass. This piece of glass was fused on the emanation tube some time before the experiment was begun. The paraffin disk with the emanation tube was fitted into one end of a glass cylinder of about 30 mm. height and approximately 17 mm. inside diameter. The disk was now sealed in the cylinder by passing the end of a warm glass rod around its inner circumference. As additional safety, the end of the cylinder with the disk was dipped into melted paraffin. As soon as the paraffin hardened the receptacle was ready to receive the trypsin solution to be exposed. The entire active portion of the emanation tube was surrounded by liquid. In the arrangement described, the thickness of the layer of fluid around the tube was about 8.5 mm. Since the absorption of radiation is a function of the chemical composition and the density of the substance being acted upon, and since the density of the substances used in our experiments is practically the same as water, the absorption must be essentially the same. Therefore we can safely assume the absorbing power of the solutions under discussion to be substantially the same as for water. It has been shown in this laboratory that a layer of water of the thickness stated is sufficient to absorb practically all the beta radiation emitted from the emanation tubes.

Our experimental procedure has been as follows. The trypsin solution was put into one of the containers previously described with a

[1] Failla, G., *Arch. Radiol. and Electrotherap.*, 1920, xxiv, 3.

pipette calibrated to deliver 2.95 cc. of water at 19°C. The beginning of the exposure was taken from the time observed when the pipette was half emptied. The receptacle containing the test was put into a compartment in a lead block which was kept in melting ice in a refrigerator. The refrigerator temperature was maintained between 4° and 6°C. Controls and stock solutions were kept under the same temperature conditions. At the end of the period of exposure the emanation tube was removed with forceps and the trypsin solution was thoroughly mixed. The mixing of the solution before a sample is withdrawn for a test is important because the chemical change effected by the radiations does not take place uniformly throughout the fluid. This is true for the following reasons. The beta rays consist of electrons moving with different velocities, the penetrating property of which is a function of their speed. The slower moving particles are absorbed more readily, hence a large part of these radiations will be absorbed in the layer of fluid immediately adjacent to the emanation tube. Also there is a decrease in the intensity of the radiations due to the distance factor, though to a less extent than would be the case for a point source of radiation since the inverse square law does not hold for the conditions described for the experiments. It follows from these facts that the greatest amount of chemical change takes place in a thin layer around the tube. We do not assume, however, that the chemical change found in different parts of the solution is brought about only by the radiations which are absorbed there, because the effect of diffusion in the solution is important.

After mixing the radiated solution a sample of 0.5 cc. was withdrawn and the active trypsin present was measured by Northrop's viscosity method.[2] The sample of trypsin solution is added to 25 cc. of 3 per cent gelatin of about pH 7.4. The gelatin is kept in a water bath at 34°C. 10 cc. of the gelatin-trypsin mixture are pipetted into a viscosimeter contained in the same bath and the time of outflow between two points on the viscosimeter pipette is measured in seconds by a stop-watch. Observations of this kind are made at frequent intervals of time. The times of outflow in seconds are plotted as ordinates against the time period of observation as abscissæ. Then

[2] Northrop, J. H., and Hussey, R. G., *J. Gen. Physiol.*, 1922–23, v, 353.

a smooth curve is drawn through the coordinate points. The reciprocal of the time required for a change in 20 per cent of the extrapolated zero point is taken as a measure of the activity of the enzyme solution. As an arbitrary unit of measurement the value of one unit is assigned to a trypsin solution which gives the experimental end-point in 1 hour. In our experiments the original value of the active trypsin in a solution (Q_o) to be radiated has been the mean of several determinations made before, during, and after the observations. The value of the active trypsin remaining in the solution after exposure to radiation has been the mean of two determinations.

The trypsin solutions have been prepared by mixing 1 gm. of Fairchild's powdered trypsin in 50 cc. of water. This mixture was allowed to stand 1 hour, then filtered, and refiltered. For the test 1.5 cc. of the resulting solution are made up to 80 cc. with 0.2 M NaCl. This dilution yields about three units of active trypsin per 0.5 cc. This value remained constant, within the limits of measurement our method permits, throughout the period of any experiment. The dilutions were made from fresh preparations for each experiment.

EXPERIMENTAL.

Having observed a striking qualitative destructive effect on a diluted trypsin solution,[3] we proceeded to determine whether we could establish a quantitative relation between this chemical change and the radiations producing it. In order to make the presentation of our experiments clear it is necessary to consider briefly some underlying factors involved in the measure and use of radium emanation as employed by us. The practical unit of measure for the quantity of emanation is the millicurie. By definition a millicurie is that amount of emanation in equilibrium with 1 milligram of radium. Radium emanation is constantly undergoing decay so that its value, when separated from its parent, radium, is constantly decreasing. In dealing with the chemical action of its radiations when the emanation is separated from radium one has to consider the influence of this

[3] We found that concentrated solutions were not affected under the conditions of irradiation employed by us. This is apparently due to the fact that the combined trypsin is not affected.

decay on the change under observation. Since this decay follows a well established law, it can be corrected for. This may be done in two ways. Observations can be made at different time intervals, as is the usual custom for investigating a chemical reaction, and then a correction for the decay calculated. This is a cumbersome procedure. A more simple method of procedure is possible because of the following relations. For any given source of emanation, the radiant energy is proportional to the quantity in millicuries. If the source is acting for a certain length of time the radiant energy, which is emitted during that time, may be represented by a value determined by the product of the average intensity (E_a), expressed in millicuries, and the time in hours (t) elapsed; *i.e.*, $E_a t$ = a certain value termed millicurie hours which is an energy unit (W). This unit is somewhat analogous to kilowatt hours employed in electrical measurements. It may further be stated that the millicurie hour measure is represented by the area under the curve

$$E = E_o e^{-\lambda t} \qquad \text{I}$$

i.e.

$$W = A = E_o \int_o^t e^{-\lambda t} dt \qquad \text{II}$$

where E_o is the initial strength of the emanation in millicuries, e is the base of natural logarithms, λ the decay constant of radium emanation, and t is the time in hours.[4] This expression integrated and solved for t with the modulus for converting natural logarithms to base 10 logarithms introduced results in the following equation

$$t = -307.1 \left[\log_{10} \left(1 - \frac{W}{133 E_o} \right) \right] \qquad \text{III}$$

It is a simple procedure now to substitute any desired value for W and solve for t, the period of exposure.

An experiment was made under the conditions that the product $E_a t$ was constant. Equal volumes of dilute trypsin solution giving an initial value for Q of 2.87 units were exposed, in three separate tubes prepared as previously described, to radium emanation of the following initial strengths; 44.1, 85.4, and 154.2 millicuries. The three exposures were started simultaneously. 70 was arbitrarily

[4] Failla, G., *Am. J. Roentgenol.*, 1921, viii, 674.

chosen as the constant value for the product of the two variables, so it may be said that the solutions were given 70 millicurie hours exposure. This experiment was repeated twice, after intervals of 2 and 20 hours respectively. Q, determined for the control solution before the last observation was made, gave a value of 2.73 units. The results are shown in Table I. The mean value of Q for all determina-

TABLE I.

Effect on Dilute Trypsin Solutions When $E't' = Et$.

Tube No.	Average millicuries.	Period of exposure.	Units observed after exposure.
		hrs.	
1	154.2	0.454	2.39
2	85.4	0.819	2.47
3	44.1	1.588	2.42
Average.			2.43
1	151.9	0.461	2.39
2	84.1	0.832	2.40
3	43.5	1.621	2.34
Average.			2.38
1	132.2	0.530	2.31
2	73.2	0.956	2.36
3	37.8	1.852	2.35
Average.			2.34

tions after exposure is 2.38, *a.d.* $\pm$ 0.03 units. The mean values of Q for each tube and the deviation of these from the mean of all values of Q (2.38 units) are as follows:

Tube 1. 2.36, -0.02; Tube 2. 2.41, $+0.03$; Tube 3. 2.37, -0.01.

As these deviations are within the precision of the method employed, the results indicate that the amount of trypsin decomposed in dilute solutions by the radiations (beta and gamma) from radium emanation is a function of the product of the two variables, millicuries and hours, and the concentration of trypsin.

Further investigation of this function was made in the following experiment. Equal volumes of a dilute solution of trypsin giving an initial value of 3.29 units for Q were exposed successively to one

emanation source, having an initial value of 164 millicuries, for periods of time so that the exposures were 50, 100, 200, and 300 millicurie hours respectively. The values of Q observed after these exposures were plotted as ordinates against the energy unit millicurie hours as abscissæ. A smooth curve was drawn through these coordinates which appeared to represent an exponential function. If this is true the logarithm of Q plotted against the energy unit (W) should represent

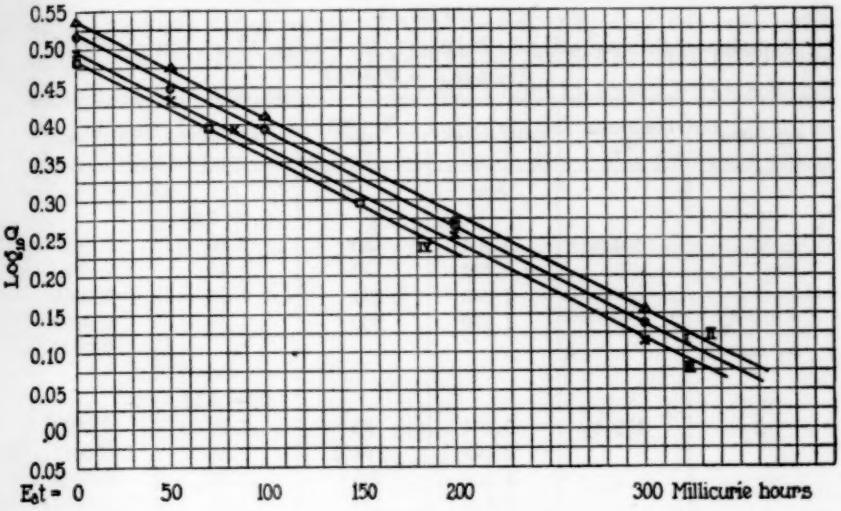


FIG. 1.

a straight line. This was found to be the case. The graphic representation of this is shown in Fig. 1 and the formulation is as follows.

$$\frac{\Delta \log Q}{\Delta W} = -k \tag{1}$$

where Q is the concentration of active trypsin observed at any millicurie hour period W, and k is a constant. From this it follows that

$$\log Q = -kW + C \tag{2}$$

where C is a constant.

When $W = 0, Q = Q_0$

$\therefore \log Q_0 = C$

Substituting this value of C in equation (2) we have

$$\log Q = -kW + \log Q_o, \qquad \text{or} \qquad Q = Q_o e^{-kW} \tag{3}$$

Solving for k we get

$$k = \frac{1}{W} \log \frac{Q_o}{Q} \tag{4}$$

For the experiment described the average value for k was 0.00293 (mc. hrs.)$^{-1}$. A similar experiment was made with a different emana-

TABLE II.

Experiments Demonstrating the Relation of the Product Et to Chemical Effect.
Average k = 0.00284.

Experiment.	Millicurie E_o	Period exposed.	Millicurie hours W	Units Q observed.	$k = \frac{1}{W} \log_e \frac{Q_o}{Q}$
		hrs.			
				Q_o = 3.29	
1	164.0	0.304	50	2.80	0.0031
		0.614	100	2.47	0.0029
		1.240	200	1.87	0.0028
		1.870	300	1.39	0.0029
Average.					0.0029
				Q_o = 3.12	
2	170.4	0.294	50	2.70	0.0027
		0.495	83.2	2.46	0.0027
		1.200	200	1.80	0.0029
		1.840	300	1.30	0.0028
Average.					0.0028
				Q_o = 3.39	
3	134.7	0.371	50	2.96	0.0029
		0.750	100	2.59	0.0028
		1.519	200	1.88	0.0028
		2.310	300	1.43	0.0029
Average.					0.0029
				Q_o = 3.03	
4	47.0	1.667	75	2.46	0.0028
		3.287	150	2.01	0.0027
Average.					0.0028

tion tube and k was found to have a value 0.00281. Two more experiments were made with results that gave satisfactory agreement with the two preceding ones. The data from these experiments are

given in Table II where it will be observed that the agreement is generally satisfactory. The value of emanation was varied in a ratio of approximately 1:4.

We can now predict the amount of Q remaining for any period covered by our observations. This fact permits a convenient method for extending the ratio of the millicurie values employed in the experiments just described. Three tests were made for this purpose. Equal volumes of trypsin solution were exposed to the radiations from three different tubes of emanation of 6.9, 8.2, and 9.9 millicuries respectively for millicurie hour periods of 138.6, 150, and 150 in the order named. The amount of Q that should be found if the relation established above would hold was calculated from equation (3) using the average k from all experiments (Table II). The following result was obtained.

$k = 0.00284.$

Tube.	Millicuries E_0	Period of exposure.	Units of trypsin.	
			Observed.	Calculated.
		hrs.		
		$Q_0 = 2.98$		
1	6.9	22.60	2.05	2.01
2	8.2	16.71	1.97	1.95
		$Q_0 = 2.87$		
3	9.9	16.10	1.84	1.89

The agreement between the calculated and observed values is satisfactory. This extends the ratio of millicurie values to about 1:25.

These results taken together permit the following statement. *The percentage of chemical change effected, as measured by determining the units of Q remaining after irradiation of a dilute trypsin solution by the radiations from radium emanation (beta and gamma), is a function of the product of the two variables, millicuries and hours.* This is true (1) within the range of the quantity of radium emanation employed (1:25),(2) within the limits of precision of our methods, and (3) over the extent of the decomposition of trypsin studied (about 60 per cent).

The data presented show that the percentage decomposition is directly related to the energy emitted. It does not, however, furnish any indication as to the relationship between energy emitted and the

energy utilized. It is of interest, nevertheless, to observe the amount of energy liberated, expressed in gram-calories,[4] from the emanation during the periods of the experiments made. The total energy emitted by 100 millicuries of radium emanation is 10.9 gram-calories per hour. Of this the beta and gamma radiations combined contributed only 1.08 gram-calories, 0.68 from the beta, and 0.40 from the gamma. Since we have been unable to measure any effect when the trypsin solutions are irradiated by gamma radiations alone for periods comparable to those stated in the experiments shown in Table II, we assume the decomposition to be produced mainly by the beta radiations. From the above it follows that 1 millicurie of emanation will liberate 0.0068 gram-calories per hour. In our experiments then the total energy emitted as beta radiations was 0.34, 0.68, 1.36; and 2.04 gram-calories for the periods, 50, 100, 200, and 300 millicurie hour exposures. We know that a considerable amount of this was lost so it is evident that very small amounts of energy were available.

SUMMARY.

The experiments presented suggest that the reaction studied is monomolecular. They show also that the decomposition is proportional to the concentration of trypsin and the quantity of emanation. The entire reaction may be formulated as follows.

$$-\frac{dQ}{dt} = k\,Q\,E \tag{5}$$

where k is a constant, Q the concentration of trypsin, and E the amount of emanation in millicuries. As previously stated, the quantity of emanation is decreasing as a function of time, *i.e.* $E = E_o e^{-\lambda t}$. Substituting this value for E in equation (5) we have

$$-\frac{dQ}{dt} = k\,Q\,E_o\,e^{-\lambda t} \tag{6}$$

which on integration and solution for k becomes

$$k = \frac{\lambda \log_e \frac{Q_o}{Q}}{E_o\,(e^{-\lambda t} - 1)} \tag{7}$$

but from equation II

$$W = \frac{E_o}{\lambda}\,(e^{-\lambda t} - 1) \tag{8}$$

Substituting W for its value in equation (7) we have

$$k = \frac{1}{W} \log_e \frac{Q_o}{Q} \tag{9}$$

where k is the constant for the experiments shown in Table II, where the millicurie hour measure is used.

Now if E in equation (5) were assumed constant this equation on integration and solution for Ek gives

$$K = Ek = \frac{1}{t} \log_e \frac{Q_o}{Q} \tag{10}$$

and this is the K for the curves shown in Fig. 2. It follows from this equation that in order to decompose the same amount of trypsin in

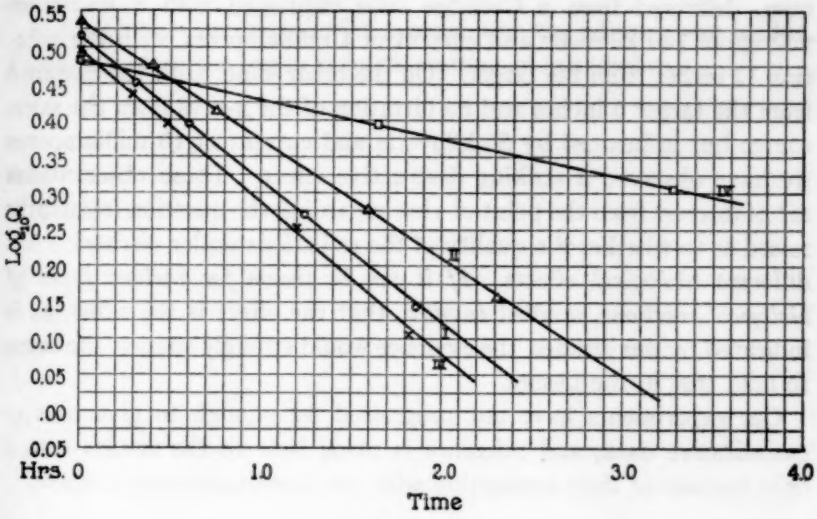


FIG. 2.

two experiments with varying amounts of E it is necessary to use such a value of t that $E't' = Et$, and this is demonstrated in the first experiment (Table I). The formulation given is analogous to an enzyme reaction occurring under conditions such that the enzyme is undergoing spontaneous inactivation. This case was originally considered by Tammann[5] and recently employed by Northrop.[6]

[5] Tammann, G., *Z. physik. Chem.*, 1895, xviii, 426.

[6] Personal communication.

As to the nature of the radiochemical decomposition studied we have no direct information, but it apparently is an irreversible reaction and in this respect is analogous to the decomposition of trypsin by heat as studied by Northrop.[7] The fact that concentrated trypsin solutions are not measurably affected tends to strengthen this analogy.

From other experiments not reported in this paper we have qualitative evidence which indicates that gamma radiations alone decompose trypsin in dilute solution. However, an exposure for a period as great as 2,000 millicurie hours produces an effect comparable to a period of about 150 millicurie hours by beta radiations. As to the effect of x-rays we have found no effect we can measure when our solutions are irradiated for 2 hours by x-rays filtered through 3 mm. of aluminum, delivered from a Coolidge tube impressed with a maximum voltage of 140 kilovolts and current of 4 milliamperes, with the solution 12 inches from the target. On the other hand, with the distance from the target 6 inches and no filtration of the x-rays from the same source but influenced by 56 kilovolts and current of 10 milliamperes we have observed a striking destructive effect. These observations are of interest from the point of view regarding the question frequently raised as to whether the quality of the radiations under discussion has different biological effects. *If it can be shown for various types of biological reactions*, studied *in vitro*, that the effect is the same, as is indicated by our studies, the evidence would strongly suggest the same to hold true in the tissues.

Our experiments have not progressed far enough to give us any quantitative data, and reference is made here to the results stated only because of their connection with our investigation as a whole.

CONCLUSION.

A quantitative study has been made of the radiochemical decomposition of trypsin by the radiations from radium emanation.

The following equation accounts quantitatively for the experimental results presented.

$$k = \frac{\lambda \log_e \frac{Q_o}{Q}}{E_o (e^{-\lambda t} - 1)}$$

[7] Northrop, J. H., *J. Gen. Physiol.*, 1921–22, iv, 261.

It follows from this that the amount of trypsin decomposed by the radiations from radium emanation depends on the concentration of trypsin present and is proportional to the quantity of emanation expressed in millicuries and to the time of irradiation expressed in hours. It would seem that it is the active or undissociated trypsin that is affected.

Evidence has been found which suggests that the beta radiations produce the decomposition observed for which the above statement holds.

Qualitative evidence has been found which suggests that x-rays, gamma rays, and beta rays produce identical effects in dilute trypsin solutions.

We wish to acknowledge with appreciation the advice given by Mr. Failla regarding the physical considerations of our studies.

MEMBRANE POTENTIALS IN THE DONNAN EQUILIBRIUM.

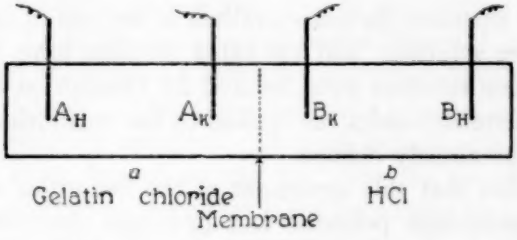
By DAVID I. HITCHCOCK.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, March 28, 1923.)

The development of Loeb's theory of the colloidal behavior of the proteins has recently been criticized by A. V. Hill.[1] This criticism seems to be based on a misunderstanding, which it is hoped the following considerations may help to clear up.

The arrangement of the experiments in question is quite clearly indicated by the diagram given by Hill, which is reproduced herewith.



(The diagram gives, however, no indication of the hydrostatic pressure due to the liquid in the manometer tube, which is a necessary condition for equilibrium. For further details of the experiments Loeb's original papers and book[2] may be consulted.) A_H and B_H represent hydrogen electrodes; A_K and B_K, calomel electrodes connected to the solutions by saturated KCl.

Loeb measured the potential difference between A_K and B_K, which he termed the observed P.D., or membrane potential. He also meas-

[1] Hill, A. V., *Proc. Roy. Soc. London*, Series A, 1923, cii, 705.

[2] Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 1922.

ured the pH of each solution separately; that is, he measured the P.D. between A_H and A_K and that between B_H and B_K. The difference between these values he termed, somewhat unfortunately, perhaps, the calculated P.D. This term is being replaced in his more recent writings by the term hydrogen electrode potential. The agreement between the membrane potential and the hydrogen electrode potential was interpreted by Loeb to indicate the applicability of Donnan's theory of membrane equilibria.

Donnan had predicted that when two solutions were separated by a membrane impermeable to one ion of one of the solutions, but permeable to other ions, then, at equilibrium, the product of the concentrations of the diffusible ions on one side of the membrane should be equal to the product of the corresponding concentrations on the other side. He further predicted that a potential difference should exist between the two solutions, and that its magnitude should be given by the relation

$$E = \frac{RT}{F} \ln \frac{x}{y}$$

where x and y represent the concentrations of any one of the diffusible ions in the two solutions, and the other symbols have their usual meaning. These relations were deduced by Donnan on the basis of the laws of thermodynamics, as applied to the equilibrium in a system of the type already defined.

Hill maintains that this agreement of the quantities which Loeb has termed membrane potential and hydrogen electrode potential "is a necessary consequence of *any* mechanism which does not offend the Second Law of Thermodynamics. . . . " This is quite true; but in this case it is just this particular mechanism of a membrane impermeable only to gelatin which has brought about the establishment of equilibrium. It had been shown repeatedly that the collodion membranes used in Loeb's experiments were impermeable to proteins but permeable to simple inorganic electrolytes. The existence of protein ions in these solutions was shown by the earlier experiments[3]

[3] Hardy, W. B., *J. Physiol.*, 1905–06, xxxiii, 251.
Pauli, W., Kolloidchemie der Eiweisskörper, Dresden and Leipsic, 1920.
Michaelis, L., Die Wasserstoffionenkonzentration, Berlin, 1914 and 1922.
Sörensen, S. P. L., *Compt. rend. trav. Lab. Carlsberg*, 1917, xii.
Robertson, T. B., The physical chemistry of the proteins, New York and London, 1918.

of Hardy, Pauli, Michaelis, Sörensen, Robertson, Loeb,[2] and others, on electrical migration and electrometric titration of protein solutions. Hence the conditions necessary for the establishment of a Donnan equilibrium were known to be satisfied; Loeb's experiments tested and verified this idea.

Hill's criticism takes no account of the fact that Loeb's experimental demonstration of the existence of a potential difference was made in a system conforming to the requirements postulated by Donnan. Since Donnan's reasoning was based on the laws of thermodynamics, of course it may be said that the experimental verification of his equation, connecting membrane potentials with ion concentrations, is simply a consequence of the laws of thermodynamics. The same might be said of the experimental verification of Nernst's formula for the E.M.F. of a concentration cell, or of almost any other of the classical experiments of physical chemistry.

Hill's reasoning to the effect that the P.D. between A_H and B_H must be zero is correct, but he seems to forget that the very condition by which this equilibrium is rendered possible is the presence of a membrane impermeable to protein ion. One is not justified in imagining, as Hill does, "that for any physical reason whatever, not *necessarily* because of the existence of a Donnan Equilibrium involving an indiffusible ion, there is a difference of potential between two solutions in equilibrium with one another." In these experiments the difference of potential must depend on the fact that the protein cannot get through the membrane, since that is the only thing that prevents the solutions from being identical at equilibrium. We have the charged or ionic protein, and the membrane impermeable to it; these are the conditions necessary for the existence of a Donnan equilibrium.

The striking facts which Loeb has discovered are the existence of a measurable difference in ion concentrations and of a corresponding measurable difference in potential between the solutions on the two sides of the membrane. These facts can be satisfactorily explained by Donnan's theory, and Loeb has shown how they can be used to explain other properties of protein solutions, notably, the effect of electrolytes on osmotic pressure. Hill has offered no alternative explanation for these facts. Loeb has explained them quantitatively; unless some better quantitative explanation can be proposed, the applicability of Donnan's theory will stand.

VALENCY RULE AND ALLEGED HOFMEISTER SERIES IN THE COLLOIDAL BEHAVIOR OF PROTEINS.

I. The Action of Acids.

By JACQUES LOEB and M. KUNITZ.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, March 19, 1923.)

I.

INTRODUCTION.

It has generally been held in the colloidal literature that different anions of the same valency have different effects on such colloidal properties of proteins as swelling, viscosity, or osmotic pressure, and that the anions could be arranged in definite series according to their influence on these properties. Such series are known under the name of the Hofmeister series. A number of authors have published such series, but the series of no two authors agree. Pauli[1] gives the following series for the influence of acids on the viscosity of blood albumin,

HCl > monochloroacetic > oxalic > dichloroacetic > citric > acetic > sulfuric > trichloroacetic acid

where HCl increased the viscosity most and trichloroacetic least.

Loeb has shown in a series of investigations that the Hofmeister series, as far as they refer to colloidal behavior of proteins, are generally the result of a methodical error; namely, the failure of the authors to measure the hydrogen ion concentration of their protein solutions or protein gels, and the failure to compare the effect of acids on the properties of proteins at the same hydrogen ion concentration of the protein solution or protein gel.[2] When this error is avoided, it

[1] Pauli, W., Kolloidchemie der Eiweisskörper, Dresden and Leipsic, 1920, 60.

[2] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 85, 247, 391.

is found that only the valency but not the nature of the anion of an acid has any direct influence on the above mentioned properties of the proteins, and that the Hofmeister ion series have in that case no real existence.

The substitution of the valency rule for the erroneous Hofmeister series gave the clue for the cause of the peculiar influence of electrolytes on these colloidal properties. There is one condition under which only the valency but not the chemical differences of anions can influence the physical properties of protein solutions or protein gels; namely, where membrane equilibria determine these properties.

Donnan[3] had shown that when a solution of two electrolytes is separated from water by a membrane permeable to all but one type of ions, an equilibrium condition results at which the molar concentration of the diffusible crystalloidal ions is not the same on opposite sides of the membrane. Suppose that there is inside a collodion bag a solution of gelatin chloride of a certain pH, then this solution contains in addition to H and Cl ions of the free HCl present in the gelatin chloride solution, positive gelatin ions and the Cl ions combined with the gelatin ions. (There is also non-ionogenic (isoelectric) gelatin, in a certain range of pH but this has no influence on the result.) Only the H and Cl ions can diffuse through the membrane, while the latter is impermeable to the gelatin ions. In such a case, according to Donnan, H and Cl ions should diffuse into the outside solution in such a way that at equilibrium the product of the molar concentrations of the pair of oppositely charged diffusible ions (in this case H and Cl) is the same on the opposite sides of the membrane. Inside the gelatin solution there exists the Cl in combination with the H of the free acid and with the gelatin. If the molar concentration of these Cl ions of the free acid inside the solution is y, the concentration of the H ions is also y. In addition there are the Cl ions in combination with the protein. Let z be the molar concentration of these Cl ions. Hence the total concentration of Cl ions inside the protein solution is $y + z$, while the concentration of H ions is y. The product of the two concentrations is $y\,(y + z)$.

In the outside aqueous solution there is only free HCl, since no gelatin ions can diffuse through the membrane. Let x be the molar

[3] Donnan, F. G., *Z. Elektrochem.*, 1911, xvii, 572.

concentration of the free H ions outside, then the molar concentration of the Cl ions is also x and the product of their concentrations is x^2. According to Donnan, equilibrium is established when

$$x^2 = y\,(y + z) \tag{1}$$

It follows from this equation that the sum of the diffusible ions must be greater inside the solution than outside, but must vary with the hydrogen ion concentration, and this variation causes the influence of electrolytes on the physical properties of proteins. Procter and Wilson[4] have shown that the influence of HCl on the swelling of gelatin can be explained quantitatively from Donnan's equation, and Loeb[5] has shown that the same is true for the influence of acids on osmotic pressure, viscosity, and membrane potentials of protein solutions.

Donnan's equation (1) is a quadratic equation when the anion of the acid is monovalent; Loeb[6] has shown that the equation becomes one of the third degree when instead of HCl a strong dibasic acid such as H_2SO_4 is added. Let x again be the molar concentration of hydrogen ions in the outside solution and y the molar concentration of the hydrogen ions inside the protein solution; then $\frac{x}{2}$ is the concentration of the SO_4 ions in the outside solution and $\frac{y}{2}$ the molar concentration of SO_4 ions of the free H_2SO_4 in the inside (gelatin) solution. The molar concentration of SO_4 ions in combination with gelatin becomes $\frac{z}{2}$. Then the equilibrium equation must be as follows:

$$x^2 \cdot \frac{x}{2} = y^2 \left(\frac{y}{2} + \frac{z}{2}\right)$$

or

$$x^3 = y^2(y + z) \tag{2}$$

If the two equations for membrane equilibria, (1) or (2), are the theoretical basis for the influence of acids on the colloidal behavior of proteins, it is clear that the valency of the anion of an acid must have

[4] Procter, H. R., and Wilson, J. A., *J. Chem. Soc.*, 1916, cix, 307.
[5] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 667, 691, 827; 1921–22, iv, 73, 97.
[6] Loeb, J., *J. Gen. Physiol.*, 1921–22, iv, 351.

a great influence, since it changes the degree of the equilibrium equation; while different ions of the same valency should all have the same influence, since the equation is the same for all anions of the same valency. Exceptions to this conclusion should be found only when the anions cause some secondary chemical or physical change in the constitution of the protein. In other words, if Donnan's theory of membrane equilibria is the true explanation of the influence of acids on the colloidal properties of proteins, it follows that only differences in the valency of the anion of the acid and no other differences in the nature of the anion of acids should, as a rule, influence those four properties of proteins which seem to depend on membrane equilibria, namely membrane potentials, osmotic pressure, swelling, and viscosity, and which constitute the typically colloidal behavior of proteins.

In former experiments Loeb had shown that this was the case. Since, however, the theory of colloidal behavior is thus intrinsically linked with the valency rule, it seemed of importance to make the proof as complete as possible, and this paper intends to offer additional evidence for the valency rule and the non-validity of the Hofmeister series for the colloidal behavior of proteins.

II.

The Necessity of Measuring the pH of the Protein Solution.

If the Donnan equation explains the colloidal behavior of proteins, it is necessary to compare the influence of acids not only for the same concentration of originally isoelectric protein but also for the same pH. The titration curves of isoelectric protein with acid show that when acid is added to isoelectric protein, protein salts are formed which are strongly hydrolyzed.[7] Hence, there exists in a solution of a protein salt an equilibrium between free dissociated acid, protein salt, and non-ionogenic protein. The relative amount of protein salt (or ionized protein) varies with the pH. At the same pH the same proportion of protein is in an ionized condition. The Donnan effect depends on the ionized portion of the protein and the valency of the anion of the acid. As long as the concentration of ionized

[7] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 85.

protein in a solution or a gel is the same, and if no salts are present, the solution will have the same osmotic pressure, viscosity, etc., regardless of the nature of the acid, except the valency of its anion (unless the anion causes a secondary chemical or physical alteration of the protein).

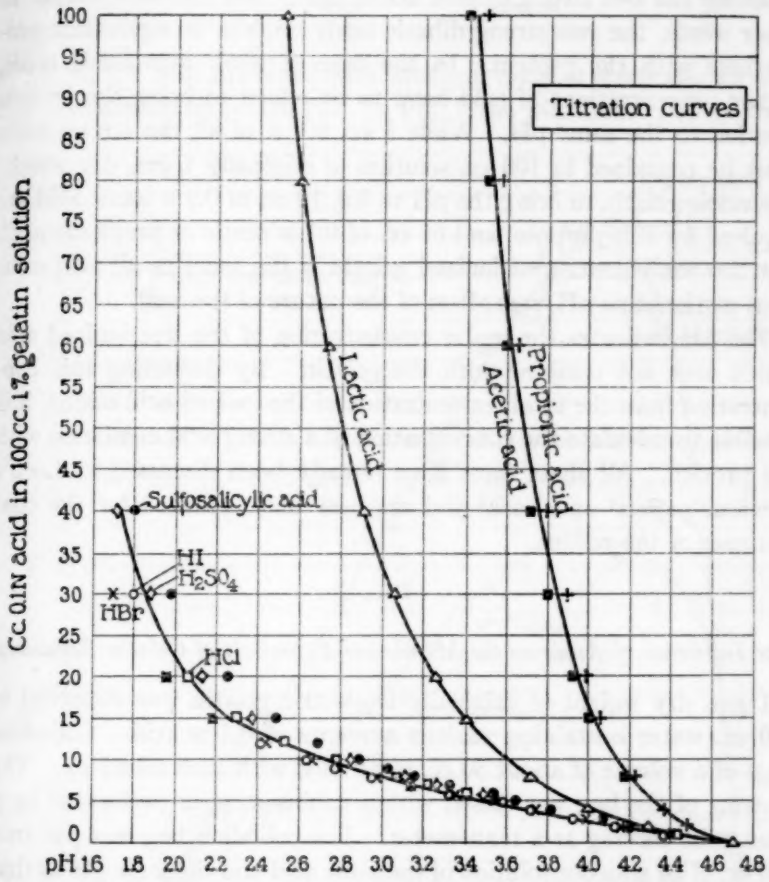


FIG. 1. Titration curves of different acids with solutions containing 1 gm. dry weight of originally isoelectric gelatin in 100 cc. The ordinates are the cc. of 0.1 N acid in 100 cc. of gelatin solution; the abscissæ the pH of gelatin solutions.

The amount of acid required to bring a 1 per cent solution of originally isoelectric protein, *e.g.* gelatin, to the same pH varies with the nature of the acid as is indicated by the titration curves in Fig. 1.

In Fig. 1 are given the titration curves of 1 gm. dry weight of originally isoelectric gelatin in 100 cc. aqueous solution for 0.1 N HCl, HBr, HI, H_2SO_4, and sulfosalicylic acid, and also for the three weak monobasic acids, lactic, propionic, and acetic. As was to be expected, the titration curves for HCl, HBr, and HI are practically identical with those for the two strong dibasic acids, H_2SO_4 and sulfosalicylic; or in other words, the two strong dibasic acids combine in equivalent proportions with the protein. In the case of weak monobasic acids, higher concentrations of acid have to be added to bring the protein solution to the same pH. While 5 cc. 0.1 N of all the strong acids must be contained in 100 cc. solution of originally 1 gm. dry weight isoelectric gelatin to bring the pH to 3.6, 10 cc. of 0.1 N lactic acid are required for this purpose, and 65 cc. of 0.1 N acetic or propionic acid. Yet the concentration of ionized gelatin is the same in all acid solutions at the same pH, regardless of the nature of the acid.

The pH indicates the molar concentration of the free ionized acid which does not combine with the gelatin. By deducting this concentration from the total concentration of the ionized acid added, it is possible to calculate the concentration of a strong acid combined with the protein. All these facts have already been discussed in Loeb's previous papers[7] and book[8] and are here only repeated for the convenience of the reader.

III.

The Influence of Acids on the Membrane Potentials of Gelatin Solutions.

1 gm. dry weight of originally isoelectric gelatin was dissolved in 100 cc. water containing various amounts of 0.1 N acid. Collodion bags of a volume of about 50 cc. were filled with such solutions. The opening of the bag was closed with a rubber stopper perforated by a glass tube serving as a manometer. The collodion bag was put into 350 cc. of an aqueous solution of the same acid and the same pH as that inside the collodion bag, but the outside aqueous solution was free from protein. The temperature was 24°C. After 18 hours or more, *i.e.* after osmotic equilibrium was reached, the membrane potential,

[8] Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 1922.

i.e. the P.D. between the protein solution and the outside aqueous solution free from protein, was measured with two saturated KCl calomel electrodes. The E.M.F. of the following cell was therefore measured in these experiments.

Hg	HgCl	saturated KCl solution	gelatin acid solution +	collodion membrane	outside aqueous solution −	saturated KCl solution	HgCl	Hg

The E.M.F. thus measured with identical calomel electrodes we shall call the membrane potential. In Fig. 2 the membrane potentials of

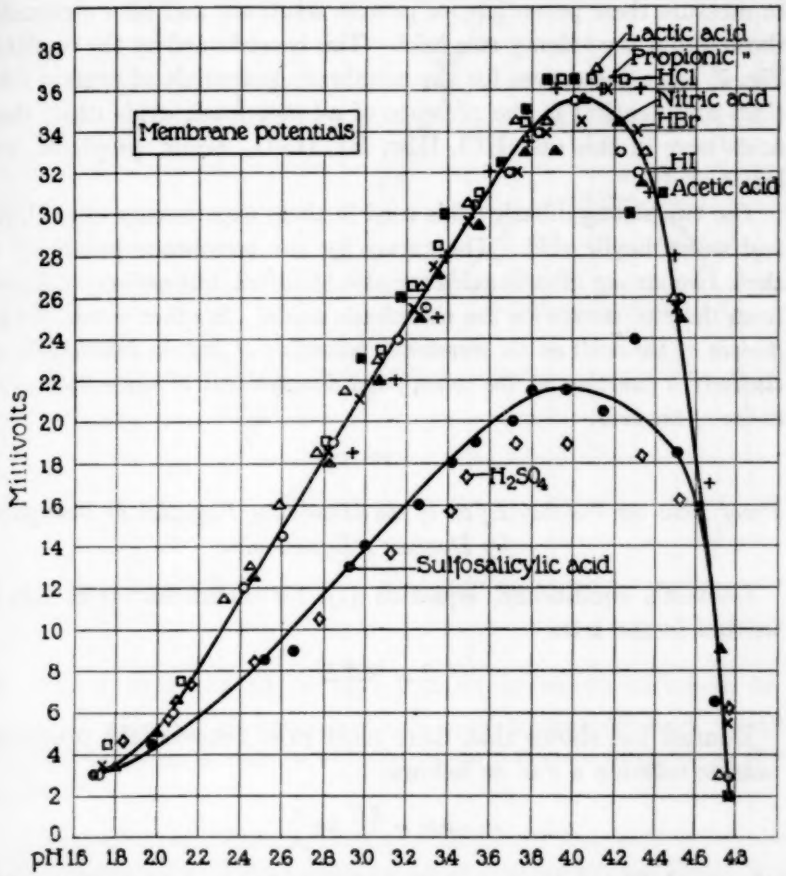


FIG. 2. Proof that only the valency of the anion of an acid influences the membrane potentials of gelatin solutions. The ordinates are the membrane potentials in millivolts; the abscissæ the pH of gelatin solutions. The membrane potentials of the seven monobasic acids are practically identical and so are the membrane potentials of the two strong dibasic acids.

the gelatin solution (*i.e.* the P.D. between gelatin solution and outside aqueous solution across the collodion membrane) are plotted as ordinates over the pH of the gelatin solution at equilibrium. (It should be stated that the pH at equilibrium is, as a rule, different from the pH at the beginning.)

The curves for the membrane potentials in Fig. 2 are as clear an expression of the valency rule and as clear a contradiction of the Hofmeister series as one could expect. It is true that nobody had claimed the validity of the Hofmeister series for the membrane potentials of protein solutions, the reason being that Loeb was the first to measure these potentials for protein solutions, and his experiments showed that the valency rule held. This is confirmed by the results in Fig. 2, since the curves for the membrane potentials of protein solutions are identical in the presence of all monobasic acids used; these acids were in this case HCl, HBr, HI, HNO_3, acetic, propionic, and lactic acid.

The two strong dibasic acids used in these experiments were H_2SO_4 and sulfosalicylic acid. The curves for the membrane potentials of these two strong dibasic acids are also identical, but entirely different from the P.D. curves for the monobasic acids. In other words, *the influence of the acids on the membrane potentials of protein solutions is exclusively a function of the valency but independent of the nature of the anion of the acid.*

IV.

Proof That the Valency Effect of the Membrane Potential Is Determined by Donnan's Equation.

Donnan's equilibrium, equation (1), for monobasic acids can be written in the form

$$\frac{x}{y} = \frac{(y+z)}{x} \tag{1}$$

Donnan has shown that there must exist between the inside and outside solution a P.D. as follows:

$$\text{P.D.} = \frac{RT}{F} \log \frac{x}{y}$$

where x is the molar concentration of hydrogen ions outside, and y the molar concentration of hydrogen ions inside. Since pH outside

is $-\log x$ and pH inside $-\log y$, the membrane potential measured with the indifferent calomel electrodes should be equal to the hydrogen electrode potential between the gelatin solution and the outside

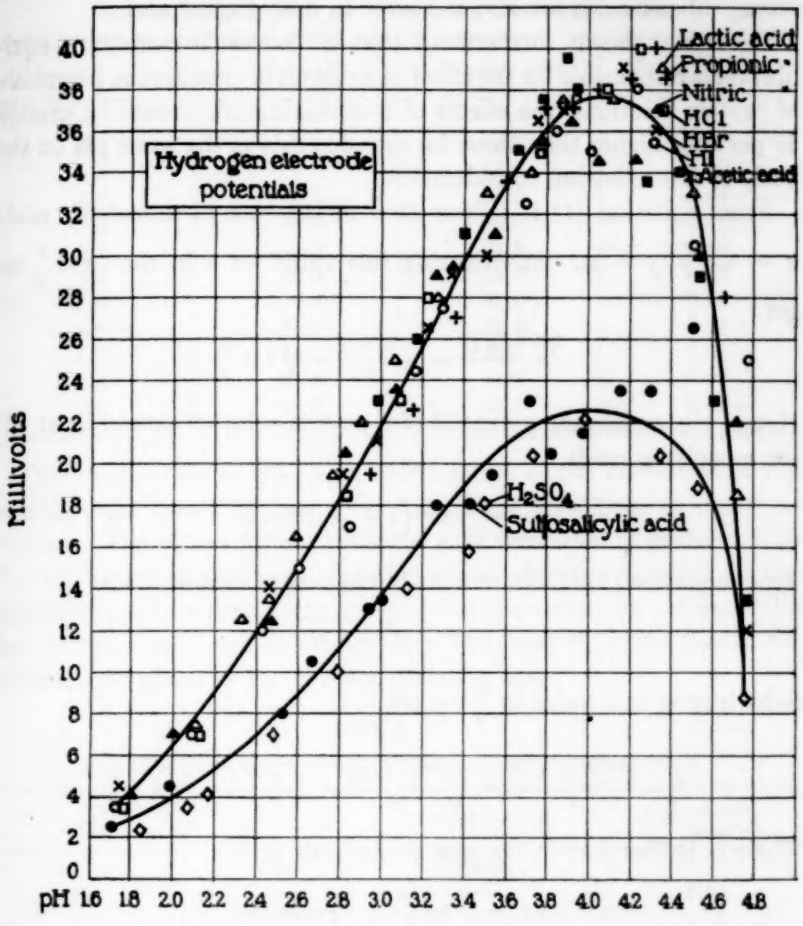

Hydrogen electrode potentials
Lactic acid
Propionic
Nitric
HCl
HBr
HI
Acetic acid
H_2SO_4
Sulfosalicylic acid
Millivolts
pH 1.6 1.8 2.0 2.2 2.4 2.6 2.8 3.0 3.2 3.4 3.6 3.8 4.0 4.2 4.4 4.6 4.8
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40

FIG. 3. Proof that the influence of acids on the hydrogen electrode potentials of gelatin solutions is identical with that on the membrane potentials as shown in Fig. 2.

aqueous solution, if the membrane equilibria are the cause of the membrane potentials. This was found to be true within the limits of the accuracy of the measurements (about 2 millivolts).

In Fig. 3 are given the hydrogen electrode potentials of the same acids as in Fig. 2. The curves for the hydrogen electrode P.D. in

Fig. 3 and the curves for the membrane P.D. in Fig. 2 are identical. The hydrogen electrode P.D. for all the monobasic acids are the same within the limits of accuracy of measurements and the P.D. for the two strong dibasic acids are also the same in both Figs. 2 and 3.

Loeb[6] had shown furthermore that if Donnan's membrane equilibrium is responsible for the effect of acids on the membrane potentials of protein solutions, the effects of monobasic acids should be exactly 50 per cent higher than those for dibasic acids at the same pH on the basis of the following consideration.

From equation (1) it follows that in the case of monobasic acids $x = \sqrt{y\,(y+z)}$. Substituting this value for x in the term $\frac{x}{y}$ we get

$$\frac{\sqrt{y\,(y+z)}}{y} = \sqrt{\frac{y+z}{y}} = \sqrt{1+\frac{z}{y}}$$

Hence, the membrane potential of a protein solution should be at 24° for monobasic acids

$$\text{P.D.} = \frac{58}{2}\log\left(1+\frac{z}{y}\right)\text{millivolts}$$

From equation (2) it follows that in the case of dibasic acids

$$x = \sqrt[3]{y^2\,(y+z)}$$

Substituting this value in $\frac{x}{y}$ we get

$$\frac{x}{y} = \frac{\sqrt[3]{y^2\,(y+z)}}{y} = \sqrt[3]{\frac{y^2\,(y+z)}{y^3}} = \sqrt[3]{\frac{y+z}{y}} = \sqrt{1+\frac{z}{y}}$$

The P.D. is therefore in the case of a dibasic acid

$$\text{P.D.} = \frac{58}{3}\log\left(1+\frac{z}{y}\right)\text{millivolts}$$

Hence, at the same pH the P.D. of gelatin sulfate must be to that of gelatin chloride as 2:3, or 0.66.

A comparison of the effects of sulfosalicylic acid with those for HCl and the other monobasic acids at the same pH in Fig. 2 shows that this is correct within the limits of experimental accuracy, except

near the isoelectric point, where the values for the monobasic acids are a trifle too high (see Table I).

TABLE I.

Membrane Potentials for Dibasic and Monobasic Acids.

pH	Dibasic acids.	Monobasic acids.	Ratio $\frac{\text{dibasic}}{\text{monobasic}}$.
	millivolts	*millivolts*	
2.4	7.6	11.4	0.67
2.6	9.6	14.8	0.65
2.8	11.6	18.0	0.64
3.0	13.6	21.6	0.65
3.2	15.8	24.8	0.64
3.4	18.0	28.0	0.62
3.6	19.8	31.0	0.64
3.8	21.2	34.2	0.62
4.0	21.6	35.5	0.61
4.2	20.8	34.8	0.60
4.4	19.2	31.0	0.62

These values are as striking a confirmation of the derivation of the valency rule from the theory of membrane equilibria as could be desired. The values for sulfosalicylic acid were used in preference to the values for sulfuric acid for the reason that a repetition of the experiment with sulfuric acid showed that the values for sulfosalicylic and sulfuric acids are in reality identical, and that the values for sulfuric acid given in Fig. 2 are a little too low.

TABLE II.

Hydrogen Electrode Potentials for Dibasic and Monobasic Acids.

pH	Dibasic acids.	Monobasic acids.	Ratio $\frac{\text{dibasic}}{\text{monobasic}}$.
	millivolts	*millivolts*	
2.6	9.9	15.4	0.64
2.8	11.4	18.6	0.61
3.0	14.0	22.0	0.64
3.2	16.6	25.6	0.65
3.4	19.2	29.4	0.66
3.6	21.0	33.0	0.64
3.8	22.0	36.0	0.61
4.0	22.6	37.5 uncertain.	0.60
4.2		Uncertain.	
4.4	21.4	36.0	0.60

Table II shows that the 2:3 ratio holds also for the hydrogen electrode P.D. values expressed in Fig. 3.

Disregarding the values for the monobasic acids at pH 3.8 to 4.2, where the values for the monobasic acids are a little too high and not quite certain, the ratio of P.D. for the effect of dibasic acid to that of monobasic acid is as nearly 2:3 as the accuracy of the measurements permits. There can therefore, be no doubt that the membrane potentials are determined by the Donnan equilibrium, and, if this be true, the valency rule must hold and there can be no room for any Hofmeister anion series in this case.

The action of weak dibasic and tribasic acids has already been referred to and it only remains to make the statement more complete. Most weak dibasic and tribasic acids dissociate as monobasic acids below a certain pH. H_3PO_4 dissociates as monobasic acid below pH 4.7 and it had been shown that in this range of pH the influence of H_3PO_4 on membrane potentials (as well as osmotic pressure, swelling, and viscosity) is identical with that of HCl or any other monobasic acid if compared for the same pH of the protein solution or gel.[8] Oxalic acid dissociates as a monobasic acid below pH 3.0 and it had been shown that for pH of 3.0 or less the influence of the oxalic acid on the properties mentioned is like that of HCl. Above pH 3.0 the second H ion of the oxalic acid begins to dissociate and the relative number of dibasic anion increases with a further increase of pH and hence the depressing effect of the dibasic anion is felt more and more the higher the pH.[8]

After these remarks, the effect of succinic, citric, and tartaric acids on the membrane potentials as plotted in Fig. 4 is easily understood. All three acids act like HCl below pH 3.0, *i.e.* the curve representing the influence of these three acids on the membrane potential coincides with that for HCl, but not with that for H_2SO_4, which means that all these acids dissociate for pH < 3.0 as monobasic acids, and furthermore, it is clear that these weak dibasic acids behave as the valency rule demands.

Above pH 3.0 the curves for succinic, citric, and tartaric acids are lower than the curve for HCl but considerably higher than that for H_2SO_4, which means that at a pH > 3.0 the second H ion begins to be split off, and the more the stronger the acid. Thus in the case of

the weak succinic acid only a very small percentage of molecules dissociates as dibasic acid and the same may be said for citric acid,

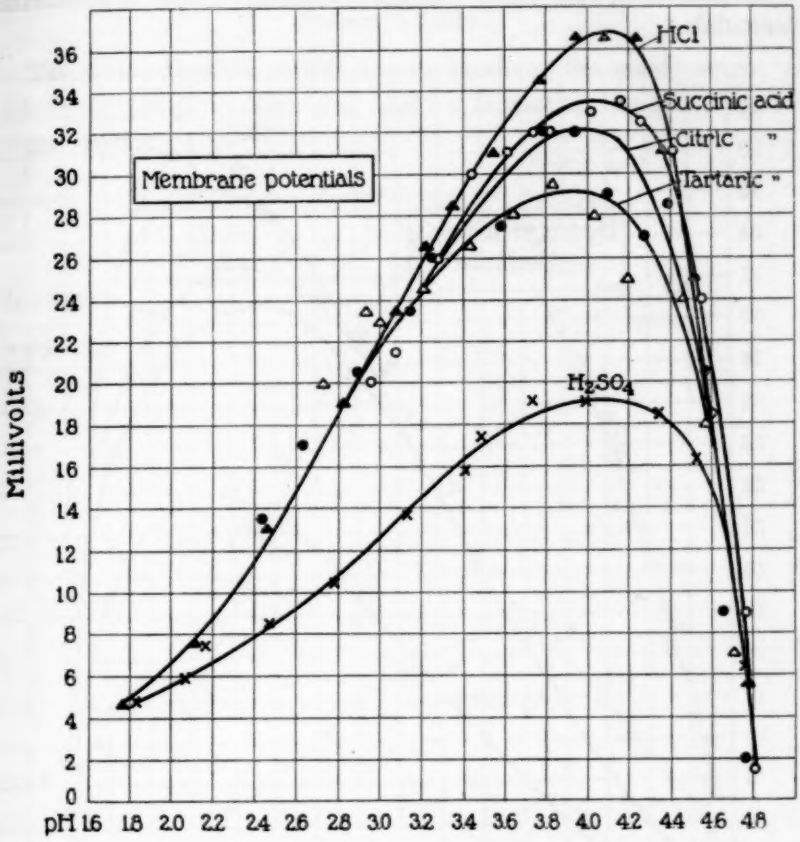


FIG. 4. Influence of weak dibasic and tribasic acids on the membrane potentials of gelatin solutions.

while a greater percentage of tartaric acid molecules dissociates as dibasic acid between pH 3.0 and 4.7. These experiments might almost be used as a criterion for the mode of dissociation of weak dibasic and tribasic acids.

Fig. 5 gives the influence of the same acids on the hydrogen electrode potentials. It is obvious that the membrane potentials observed

with the indifferent calomel electrodes are within the limits of accuracy of measurements and calculations identical with the hydrogen electrode potentials, thus confirming again Donnan's theory of membrane potentials.

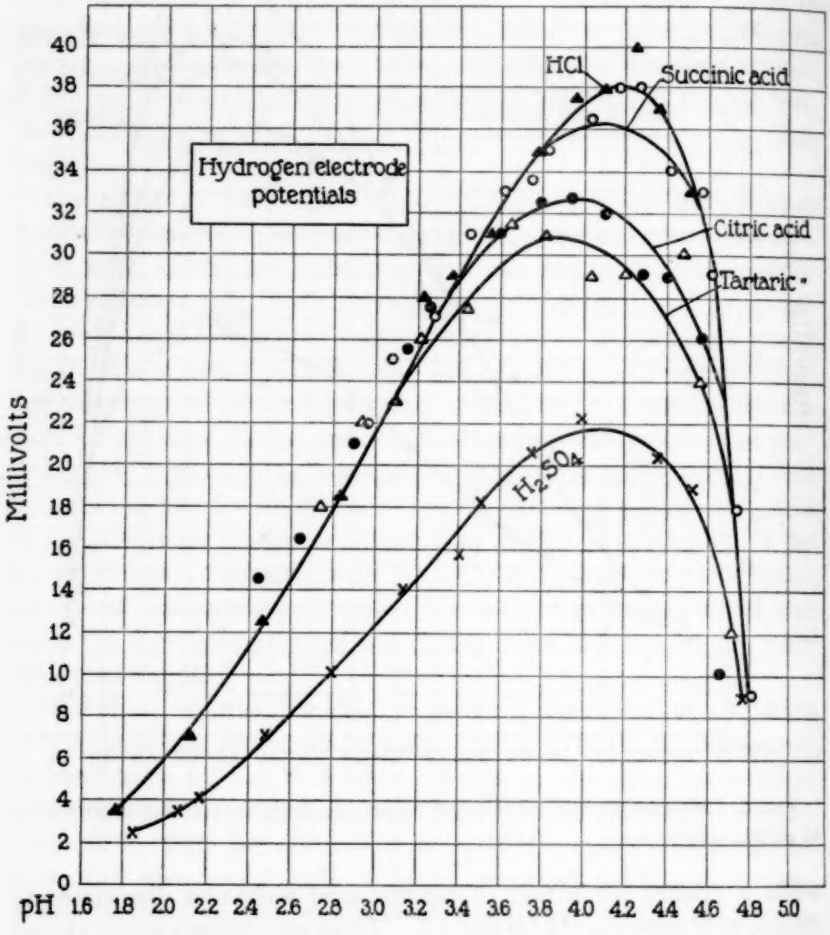


FIG. 5. Influence of weak dibasic and tribasic acids on the hydrogen electrode potentials of gelatin solutions. Notice identity of curves in Figs. 4 and 5.

All these experiments leave no doubt of the experimental and theoretical correctness of the valency rule for membrane potentials and the non-validity of the so called Hofmeister series.

V.

The Valency Effect in the Influence of Acids on the Osmotic Pressure of Protein Solutions.

The same experiments which were used for the measurement of membrane potentials were also used for measuring the osmotic pres-

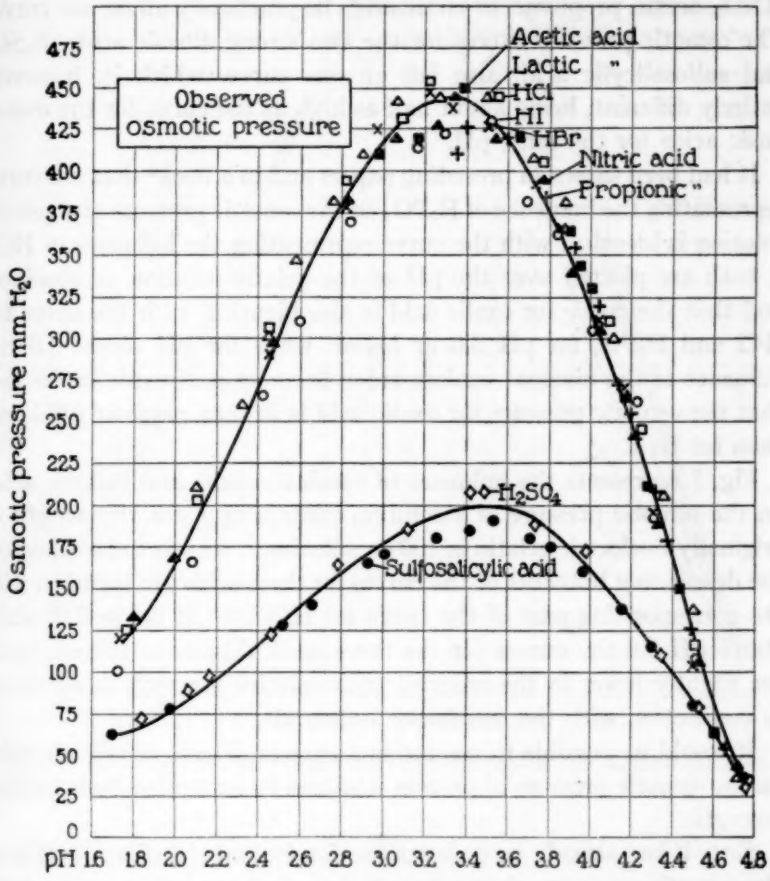


FIG. 6. Proof of valency rule for the influence of acids on the osmotic pressure of gelatin solutions. The influence of seven monobasic acids on the osmotic pressure of gelatin solutions is the same and about twice as high as that of the two dibasic acids.

sure of gelatin solutions containing 1 gm. dry weight of originally isoelectric gelatin in 100 cc. of water made up with various acids. The

results are contained in Fig. 6. The ordinates are the observed osmotic pressures in terms of mm. of a column of water, and the abscissæ are the pH of the gelatin solution at equilibrium. The result is so striking that it leaves no doubt that the valency rule is correct. All the monobasic acids influence the osmotic pressure in exactly the same way; and the values for HCl, HBr, HI, HNO_3, acetic, propionic, or lactic acids lie practically all on one curve. The osmotic pressure curves for the two strong dibasic acids, H_2SO_4 and sulfosalicylic acid, also fall on one curve, which is, however, entirely different, being about half as high as the curve for the monobasic acids for the same pH.

It had been shown in preceding papers and in a book[8] that the curve representing the influence of H_3PO_4 on the osmotic pressure of a gelatin solution is identical with the curve representing the influence of HCl, if both are plotted over the pH of the gelatin solution as abscissæ; and that the curve for oxalic acid is also identical with the curve for HCl and H_3PO_4 for pH 3.0 or below, while for pH above 3.0 the influence of the bivalent oxalate anion becomes noticeable in the fact that the osmotic pressure for oxalic acid is in that range of pH lower than for HCl.

Fig. 7 represents the influence of succinic, citric, and tartaric acids on the osmotic pressure of a solution containing 1 gm. dry weight of originally isoelectric gelatin in 100 cc. solution. As was to be expected, the descending branches of the curves for these acids are identical with the corresponding part of the curve for HCl for pH below 3.0, while above pH 3.0 the curves for the three weak dibasic or tribasic acids are slightly lower in the order of their relative strength as discussed in connection with the membrane potentials.

It would be possible to use the influence of dibasic or tribasic acids on the osmotic pressure of protein solutions to determine their relative strength.

Since it had already been shown previously that the effect of acids on the osmotic pressure of protein solutions is due to the excess of the molar concentration of diffusible ions inside the protein solution over that in the outside solution caused by the Donnan equilibrium, it is mathematically necessary that there should be only an effect of the valency of the anion of the acid on the osmotic pressure but no effect

of the nature of the anion except the valency. Figs. 6 and 7, in addition to the data already published, prove beyond doubt the correctness of this statement.

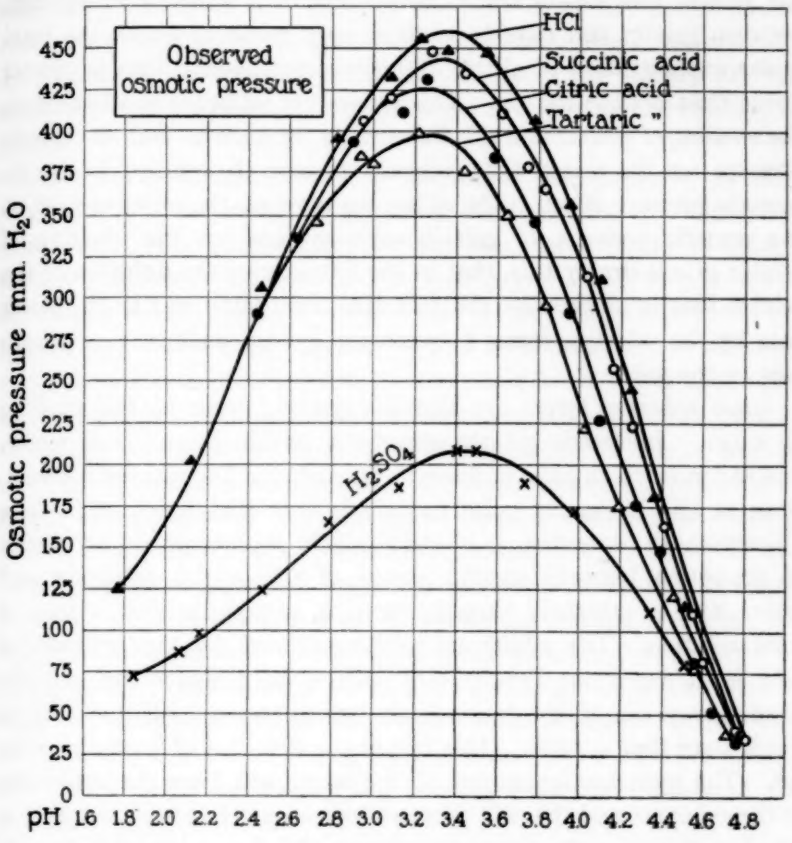


FIG. 7. Influence of weak dibasic and tribasic acids on the osmotic pressure of gelatin solutions.

VI.

Swelling.

Procter and Wilson[4] have shown that the influence of HCl on swelling of gelatin is a purely osmotic effect. The acid combining with the gelatin causes salt formation, the gelatin ions being pre-

vented from diffusing by the cohesive forces between the gelatin ions or molecules of the gel. Since the gel is freely permeable to water and crystalloidal ions, such as H and Cl, the non-diffusibility of the gelatin ions causes the establishment of a Donnan equilibrium between gelatin and outside solution, as a result of which the total molar concentration of all the diffusible crystalloidal ions is greater inside than outside the gel. This causes the influence of the acid on the swelling of gelatin and this influence is the same as that on osmotic pressure for the reason that influence of acid on swelling is also an osmotic pressure effect. The difference between the effect of acid on the osmotic pressure of gelatin solutions and on the swelling of gelatin gels is simply this, that in the former case the diffusion of the gelatin ions is blocked by the collodion membrane and in the latter case by the cohesive forces between the gelatin molecules or gelatin ions of the gel.

These cohesive forces are also the limiting force to the swelling of a gel. Isoelectric gelatin absorbs a certain quantity of water, due to forces which have nothing to do with the Donnan equilibrium, since at the isoelectric point protein is only slightly ionized. The absorption of water by isoelectric gelatin is determined by forces of attraction between certain groups of the gelatin molecule and water, and is primarily though, perhaps, not exclusively a case of solid solution. The additional swelling caused by the addition of acid is, however, as Procter and Wilson have shown, an osmotic phenomenon due to the excess in the concentration of H and Cl ions inside over that outside. This causes the diffusion of water into the gel. The hydrostatic pressure of the water will force the molecules of the gel apart and this will cause an increase in the forces of cohesion which will oppose the further swelling. To give an idea of the difference between the swelling of isoelectric gelatin and that due to the influence of acid, it may be stated that while 1 gm. dry weight of powdered isoelectric gelatin absorbed about 7 gm. of water, the same gelatin when under the influence of an acid with monobasic anion absorbed about 35 gm. of water at pH 3.2 or 3.0 where the swelling is a maximum. The forces of cohesion between the molecules or ions of the gel may be modified by the solute, *e.g.* the anion of the acid, and when this happens, the pure osmotic pressure effect, due to the

Donnan equilibrium, may not be observed. This was noticed in the effect of acids on the swelling of casein, where it was found that swelling occurs in HCl or HNO_3 but not in trichloroacetic acid. These secondary effects of the anion of the acid or of the undissociated acid on the cohesion of the gel are slight and negligible in the case of a gel of gelatin, and for this reason the validity of the valency rule can easily be demonstrated for the influence of acids on the swelling of gelatin.

The most accurate method for determining the degree of swelling of gelatin is by weighing the gelatin at the beginning and end of the experiment. The earlier experimenters used large blocks of gelatin for the weighing experiment, but in that case it requires days before the maximum of swelling is reached and in the meantime some of the gelatin may have been dissolved. The solution effect of different acids on gelatin is probably not the same and in solubility the nature of the anion may play a considerable rôle. It is therefore obvious that experiments on the swelling of blocks of gelatin cannot well be used for theoretical conclusions. We used powdered particles of gelatin of a definite size of grain; namely, particles which went through a sieve with mesh of $\frac{1}{30}$ but not through mesh $\frac{1}{60}$ of an inch. It was found that such particles reach the maximal swelling in 2 hours. By making the experiment at 15°C. the loss by solution of the gelatin was less than 5 per cent. After the acid had acted for 2 hours at 15°C. on the gelatin, the latter was put on a filter, the solution was allowed to drain off, and the weight of the gelatin was determined. In this way better results could be obtained than with the older method of using a solid block or by estimating the swelling from the volume of the powdered gelatin.

A large quantity of powdered gelatin of the size of grain as stated was brought to the isoelectric point in the way described by Loeb.[8] 8 gm. of the wet isoelectric powdered material contained 1 gm. dry weight of isoelectric gelatin. This mass of powdered isoelectric gelatin in equilibrium with water served as stock material and was kept moist in an ice chest at about three degrees centigrade. Equal portions of 8 gm. each of the stock gelatin, each containing about 1 gm. dry weight of isoelectric gelatin, were put into beakers and allowed to stand for about 18 hours in a moist chamber of 15–16°C. The

8 gm. portions of gelatin were then added each to 150 cc. of H_2O containing various amounts of 0.1 N acids of 15°C. and allowed to stand, with frequent stirring, for 2 hours at 15°C. The gelatin was then removed from the outside solution by filtration through cotton-wool in weighed funnels. The liquid was allowed to drain off completely and the weight of gelatin was determined. The gelatin was afterwards melted at 50°C., cooled to 25°, and its pH was measured electrometrically. All operations, except pH measurements, were done in a constant temperature room at about 15°C. A control consisting of 9 cc. of 0.1 N HCl per 150 cc. of H_2O was used with each

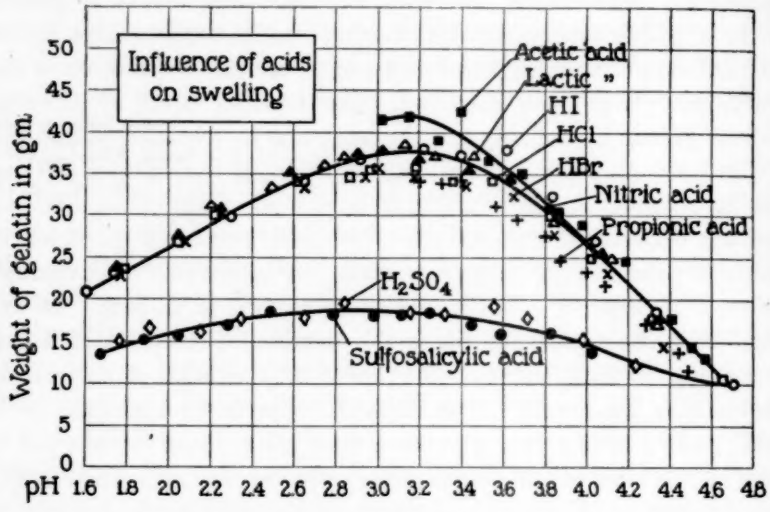


FIG. 8. Proof of valency rule for the influence of acids on the swelling of gels of gelatin. The influence of the seven monobasic acids is (aside from slight secondary effects of acids presumably on the cohesion of the gel) the same and considerably higher than that of the two dibasic acids.

series. The pH of the gelatin of the control was 3.19 and the weight of the control varied mainly between 34 and 36 gm., the extreme variations being 32.2 and 37.8 gm.

Fig. 8 gives the results with different acids. The abscissæ are the pH of the gel at the end of the experiment, while the ordinates are the weight of the gelatin at the end of the experiment. All the values for the influence of the six monobasic acids, HCl, HBr, HI, HNO_3,

propionic, and lactic acid on swelling lie on the same curve within the limits of the accuracy of the experiments, with a maximal weight of about 36 gm., which is inside the variations for the controls with HCl referred to. Only acetic acid gives a slightly higher maximal value of about 42 gm. at pH 3.2. This had been observed by Loeb before. The abnormal behavior of acetic acid does not occur in either membrane potentials or osmotic pressure, where the effects are due to isolated gelatin ions. The suspicion is therefore justified that the excessive effect of acetic acid on swelling is due to a diminution of the cohesion of the gel caused by the high concentration of acetic acid required to bring the pH to 3.2 or 3.0. This idea had already been suggested by Loeb.[8]

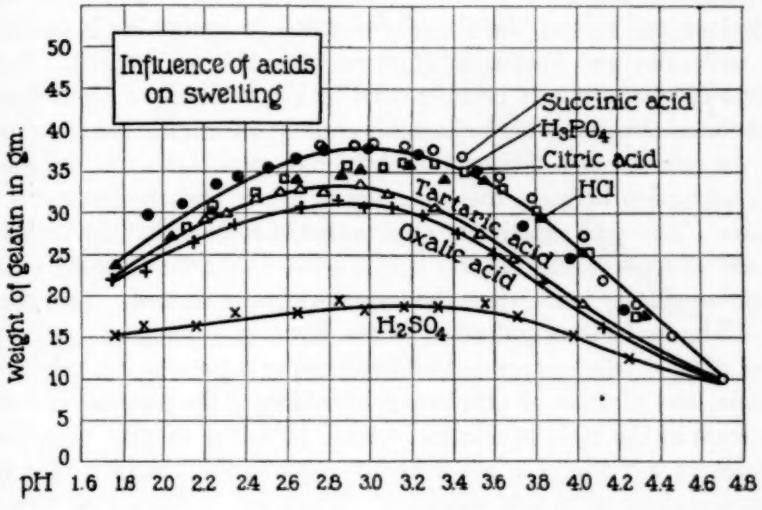


FIG. 9. Influence of weak dibasic and tribasic acids on swelling.

On the other hand, the strong dibasic acids, H_2SO_4 and sulfosalicylic acid, also act alike but cause a maximal weight of only 18 gm., which is about one-half of the maximal weight of the gelatin under the influence of HCl. This ratio of 1:2 for dibasic and monobasic acids is about the same as that observed for the valency effect of anions in the case of osmotic pressure. The maximum lies at a pH of about 3.0 to 3.2 of the gel.

These experiments show that only the valency but not the chemical nature of the anion of the acid influences the swelling of gelatin.

Fig. 9 shows the effect of weak dibasic and tribasic acids on swelling. From what has been said concerning the electrolytic dissociation of these acids it is obvious that their effect on swelling is also as clearly a confirmation of the valency rule as is their action on membrane potentials and on osmotic pressure.

VII.

Viscosity.

Loeb has shown that in the case of colloids we must discriminate between two sources of viscosity;[5] first, the ordinary viscosity of true solutions, and second, the viscosity of solutions containing in addition to molecules and ions solid aggregates capable of swelling. Both forms of viscosity may be influenced by the addition of electrolytes, but the mechanism of the influence is entirely different in the two cases. In the case of true solutions, the addition of electrolytes may influence the attraction between the molecules of solute and the molecules of water. This may explain the observation of Reyher that the viscosity of the solution of salts of fatty acid is greater than the viscosity of the solution of fatty acids, since the former are more ionized. This effect is of a low order of magnitude. When, however, submicroscopic particles of solid jelly are present in a solution of a protein, *e.g.* gelatin or casein, the addition of acid causes a swelling of the particles and this increase in the ratio of relative volume of solute to that of solvent causes an increase of a much higher order in the viscosity than the increase observed in true solutions. Moreover, in this second form of viscosity due to swelling we are dealing with a phenomenon which depends on the Donnan equilibrium. Hence in this case the valency rule should apply, while in the influence of acids on the viscosity of solutions containing no solid particles, the chemical nature of the anion of an acid might be expected to be of great influence. Since a solution of gelatin contains isolated molecules of gelatin as well as suspended particles, both forms of viscosity will be influenced by acids. But the influence of acid on the swelling of the submicroscopic particles of jelly causes a much greater increase of viscosity than the

influence of acid on the isolated molecules so that we may neglect the latter effect.

The method of procedure was as follows. A stock solution of 2 per cent isoelectric gelatin was made up and kept in the ice chest at about 3°C. A portion of the stock of the 2 per cent isoelectric gelatin was liquefied by heating to about 45°C. and then kept warm (at about 35°C.) during each experiment in order to avoid solidification. 25 cc. of the 2 per cent isoelectric gelatin were pipetted into a 50 cc. graduated flask, the required amount of acid added from

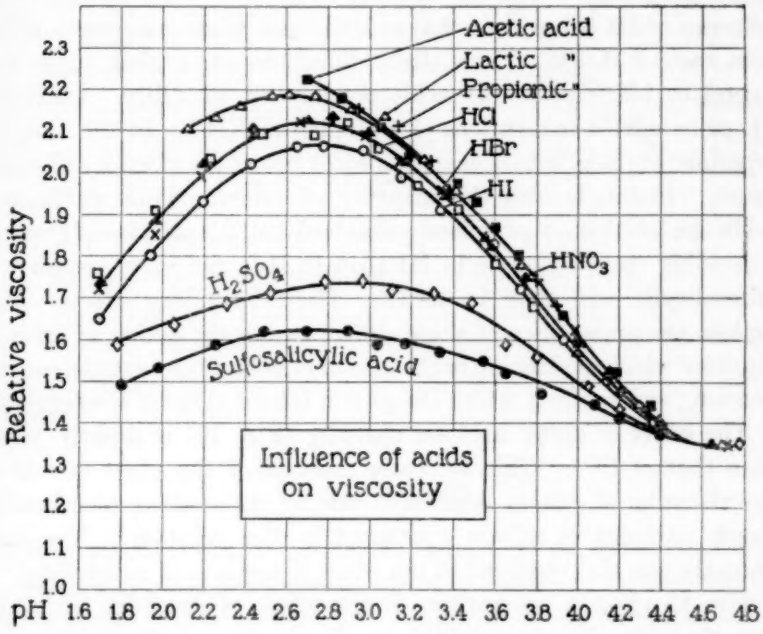


FIG. 10. Proof of the valency rule for the viscosity of gelatin solutions for seven monobasic and two dibasic acids.

a burette, and the volume was then made up to 50 cc. with H_2O. The mixture was heated in a hot water bath to 45°C., rapidly cooled in ice water to 24°C., and the viscosity was immediately measured at 24°C. by means of an Ostwald viscometer.

Fig. 10 gives the influence of the seven monobasic acids, HCl, HBr, HI, HNO_3, lactic, acetic, and propionic acids, and of two strong dibasic

acids, on the relative viscosity of gelatin solutions as described. The validity of the valency rule is obvious. The seven monobasic acids act almost alike, differing entirely from the action of the two strong dibasic acids, H_2SO_4 and sulfosalicylic. The curves are similar to the curves of swelling in Fig. 8 and of osmotic pressure in Fig. 6. This finds its explanation in the fact that this form of viscosity is due to the swelling of particles and that the influence of acids on swelling is an osmotic phenomenon, as demonstrated by Procter and Wilson.

There are, however, some slight discrepancies between the curves for swelling (Fig. 8) and for viscosity (Fig. 10). Thus in Fig. 10 the influence of HI is less than that of HCl, that of sulfosalicylic acid less than that of H_2SO_4. These slight differences are probably due to a secondary influence of the acids possibly on solubility. Gelatin is *e.g.* more soluble in solutions of NaI than of NaCl. In the viscosity experiments the gelatin is heated to 45°C. before the viscosity measurements. In this heating the majority of submicroscopic particles of gel in the solution are completely dissolved but this solution of particles is possibly more complete in HI than in HCl and more complete in sulfosalicylic acid than in H_2SO_4. These secondary effects would explain the appearance of slight differences in the action of acids on viscosity which are lacking in the case of membrane potentials, osmotic pressure, and swelling, where the gelatin is kept at lower temperatures.

The effect of acetic acid on viscosity (Fig. 10) is slightly higher than that of HCl. This was to be expected if the effect of acid on the viscosity of gelatin solution is due to the swelling of submicroscopic particles of gelatin contained in the solution. The same anomaly was also observed in the effect of acetic acid on swelling.

Fig. 11 gives a comparison of the effect of succinic, citric, and tartaric acids with that of HCl on the viscosity of gelatin solutions. The effects differ but slightly for the four acids.

Summarizing the results of the previously published experiments of Loeb and the results of the new experiments, we may say that they show that only the valency of the anion of the acid and none of the other properties of the anion influence the membrane potentials, osmotic pressure, swelling, and viscosity of gelatin; and that, moreover, this result was to be expected theoretically since the influence of acids on the four properties of gelatin is determined by the Donnan equi-

librium which varies with the valency but not with the other properties of the anions of acids.

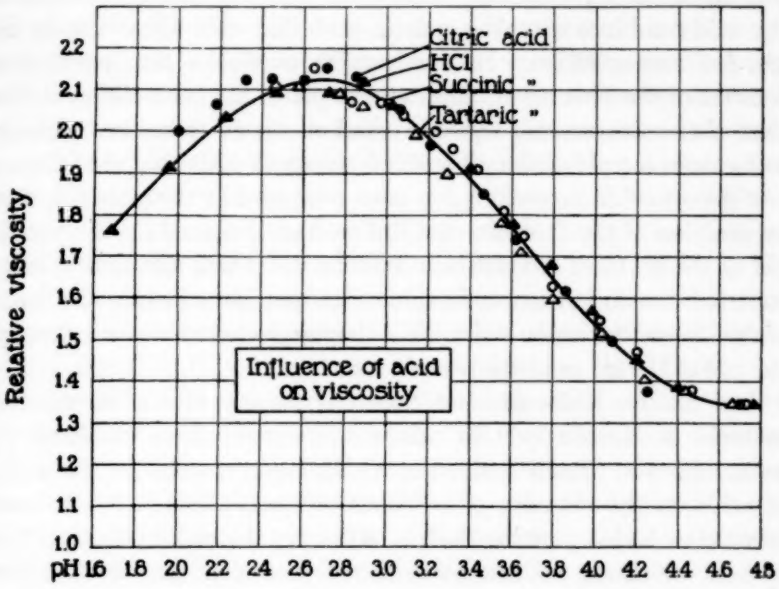


FIG. 11. Influence of weak dibasic and tribasic acids on the viscosity of gelatin solutions.

VIII.

Critical Remarks.

The conclusions reached in the older papers on the influence of acids on swelling, osmotic pressure, and viscosity are due to the fact that the effects of different acids were not compared at the same pH of the protein solution or the gel after equilibrium was reached, but, as a rule, for identical quantities of acid added to the water into which the gel was put. The effects of the same acid were therefore not compared at the same concentration of ionized gelatin in the protein solution or gel (or for the same pH of the gel) but at entirely different pH of the solution or gel. This led to error, so that the nature of the anion of an acid aside from its valency seemed to have an effect, which was in reality the effect of differences in the pH of the gel. Kuhn[9] in a paper published as late as 1921 calculates the H^+ ion concentration of protein

[9] Kuhn, A., *Kolloidchem. Beihefte*, 1921–22, xiv, 147.

gels from Kohlrausch's tables as if the pH in the presence of gelatin were the same as that of acid in water free from gelatin, omitting the consideration of the fact, proved by the titration curves, that part of the acid combines with the gelatin and that this alters the hydrogen ion concentration. He furthermore overlooks the fact that on account of the Donnan equilibrium the pH of the gel is different from that of the surrounding aqueous solution. It is, therefore, necessary to measure the pH of the gel and it is wrong to state that the influence of different acids on swelling has been compared at the same pH when no mention of the Donnan equilibrium has been made and when the pH of the gel itself has not been measured. These and similar errors have led certain authors to imagine that they have proved the reality of the Hofmeister anion series, while they neglected to measure properly the pH of the gel or of the protein solutions.

And finally, Kuhn did not take the trouble to use as standard material isoelectric protein, since this would have required pH measurements. Pauli[1] arrived at his Hofmeister series for the action of acids on the viscosity of solutions of blood albumin by the same mistake as Kuhn, plotting the viscosity over the molar concentrations of acid which the solutions would have had if no protein had been present. Lillie's[10] Hofmeister series for osmotic pressure effects was based on the same error, and the same is true for all the earlier experiments on the effect of acids on the physical properties of proteins.

IX.

SUMMARY.

1. The action of a number of acids on four properties of gelatin (membrane potentials, osmotic pressure, swelling, and viscosity) was studied. The acids used can be divided into three groups; first, monobasic acids (HCl, HBr, HI, HNO_3, acetic, propionic, and lactic acids); second, strong dibasic acids (H_2SO_4 and sulfosalicylic acid) which dissociate as dibasic acids in the range of pH between 4.7 and 2.5; and third, weak dibasic and tribasic acids (succinic, tartaric, citric) which dissociate as monobasic acids at pH 3.0 or below and dissociate increasingly as dibasic acids, according to their strength, with pH increasing above 3.0.

[10] Lillie, R. S., *Am. J. Physiol.*, 1907–08, xx, 127.

2. If the influence of these acids on the four above mentioned properties of gelatin is plotted as ordinates over the pH of the gelatin solution or gelatin gel as abscissæ, it is found that all the acids have the same effect where the anion is monovalent; this is true for the seven monobasic acids at all pH and for the weak dibasic and tribasic acids at pH below 3.0. The two strong dibasic acids (the anion of which is divalent in the whole range of pH of these experiments) have a much smaller effect than the acids with monovalent anion. The weak dibasic and tribasic acids act, at pH above 3.0, like acids the anion of which is chiefly monovalent but which contain also divalent anions increasing with pH and with the strength of the acid.

3. These experiments prove that only the valency but not the other properties of the anion of an acid influences the four properties of gelatin mentioned, thus absolutely contradicting the Hofmeister anion series in this case which were due to the failure of the earlier experimenters to measure properly the pH of their protein solutions or gels and to compare the effects of acids at the same pH of the protein solution or protein gel after equilibrium was established.

4. It is shown that the validity of the valency rule and the nonvalidity of the Hofmeister anion series for the four properties of proteins mentioned are consequences of the fact that the influence of acids on the membrane potentials, osmotic pressure, swelling, and viscosity of gelatin is due to the Donnan equilibrium between protein solutions or gels and the surrounding aqueous solution. This equilibrium depends only on the valency but not on any other property of the anion of an acid.

5. That the valency rule is determined by the Donnan equilibrium is strikingly illustrated by the ratio of the membrane potentials for divalent and monovalent anions of acids. Loeb has shown that the Donnan equilibrium demands that this ratio should be 0.66 and the actual measurements agree with this postulate of the theory within the limits of accuracy of the measurements.

6. The valency rule can be expected to hold for only such properties of proteins as depend upon the Donnan equilibrium. Properties of proteins not depending on the Donnan equilibrium may be affected not only by the valency but also by the chemical nature of the anion of an acid.

VALENCY RULE AND ALLEGED HOFMEISTER SERIES IN THE COLLOIDAL BEHAVIOR OF PROTEINS.

II. The Influence of Salts.

By JACQUES LOEB and M. KUNITZ.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

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Difference between the Action of Acids and of Salts on Colloidal Behavior.

In order to understand the action of salts on colloidal behavior, it is necessary to understand the difference between the action of acids and of salts. When acid is added to a solution of isoelectric protein, the membrane potentials, osmotic pressure, and, in the case of some proteins (*e.g.* gelatin), viscosity, are first increased until a maximum is reached; and when more acid is added, the values representing these properties drop again. The action of acid on the swelling of gelatin is the same, since the swelling due to acid is merely an osmotic phenomenon. This rise and fall of the curves for these four properties finds its explanation through the following facts. The addition of acid to isoelectric protein has two opposite effects; first, a salt formation between protein and acid, which increases the concentration of ionized protein, and this causes a rise in the value of the four properties mentioned with the addition of acid; and second, the depressing influence of the anion of the acid on the value of the four properties. Both effects increase with the increase in the concentration of acid, but not at the same rate. When little acid is added to isoelectric protein, the salt formation and ionization of the protein increases at first more rapidly than the depressing effect of the anion of the acid until a point is reached where the further addition of acid increases the ionization little, and finally not at all, while the depressing effect of the anion of the acid continues to increase. This peculiar action of

the acid finds its explanation in the theory of membrane equilibria, as shown in preceding papers and a book.[1]

If this theory of membrane equilibria is correct, we should expect that, when the protein salt is a salt of the type of gelatin chloride, where the gelatin ion is positively charged, only the anion of an acid (or a salt) should influence the four properties dependent on the membrane equilibrium, namely membrane potentials, osmotic pressure, swelling, and that type of viscosity which is due to the swelling of protein particles; and furthermore, that only the valency of the anion but not its chemical nature should have an effect (unless these properties are affected in an indirect way by a change in the chemical or physical constitution of the protein by a specific acid). The validity of the valency rule has already been discussed for acids and will be discussed more fully here for salts.

The difference between the action of salts and acids on the four properties of protein is this, that while acids (and alkalies) cause ionization of isoelectric protein, salts have no such effect. When a salt is added to a solution of a protein salt, *e.g.* gelatin chloride, of a given pH, only the depressing action of the anion of the salt on these properties is noticed, but no augmenting effect of the cation of the salt. This was proved by Loeb by the following two sets of experiments. When all the protein is ionized, *e.g.* when gelatin chloride of pH 2.5 is chosen, HCl and NaCl act quantitatively alike, since now no further ionization can be produced by the acid. When, however, the pH of the gelatin chloride is higher (but below pH 4.7), the addition of little acid will cause an increase in the values of the four properties which will not be produced by the salt.[1]

The second proof consisted in the fact that if the pH of the protein solution is kept constant, salts of the type of NaCl, $CaCl_2$, and $LaCl_3$ will have an equal depressing effect on the properties of protein-acid salts for the same concentration of the Cl ions of the three salts, at the same pH of the protein solution or protein gel. This was proven for membrane potentials and osmotic pressure.[2]

[1] Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 1922.

[2] Loeb, J., *J. Gen. Physiol.*, 1921–22, iv, 752. Hitchcock, D. I., *J. Gen. Physiol.*, 1921–22, iv, 597.

If the explanation of these effects of salts on the basis of the Donnan equation is correct, it follows furthermore that only the anion of a salt should have a depressing effect on the four properties of protein-acid salts, and only the cation of a salt should have a depressing effect on the properties of metal proteinates. Loeb has already shown that this is correct[1] and it is intended to add in this paper some further experimental evidence. This was considered advisable on account of the fact that some authors continue to maintain that for the action of salts on proteins the Hofmeister series are valid. Those who believe in the validity of the Hofmeister series for the action of salts on the four above mentioned properties of proteins arrange the effect of salts on the swelling of gelatin in the following way

SO_4, tartrate, citrate < acetate < Cl < Br, NO_3 < I < CNS

where the swelling is said to be a maximum in CNS and a minimum in SO_4. "Above a certain concentration the sulfates, tartrates, and citrates cause a shrinkage of the gel of gelatin, and acetate acts in the same sense but less strongly, while the other anions cause an increasing swelling of the gel of the same concentration."[3] Loeb had shown that these and similar statements were simply due to the failure of the older authors to measure the pH of the gel and that if this quantity is properly measured with the hydrogen electrode, it is found that there exists only an effect of the valency of the anion of the salt on swelling and no effect of the chemical nature of the anion (provided the pH of the protein solution or gel is on the acid side of the isoelectric point).[1]

As far as the actions of the cations of a salt are concerned, the following statement is made. "The differences in the effects of cations are less marked; it might be possible to propose the series Li < Na < K, NH_4; then follow the alkali earths with Mg in a position between."

It is also stated that these anion and cation series are correct at a neutral reaction. Since the isoelectric point of gelatin is at pH 4.7, at neutral reaction, *i.e.* pH near 7.0, gelatin exists as metal gelatinate, the properties of which are not affected at all by the anions of a salt

[3] Höber, R., Physikalische Chemie der Zelle und der Gewebe, Leipsic, 5th edition, 1922, pt. 1, 267.

and are affected strongly by the cation of a salt. This alone shows that the anion series at pH 7.0 must be the result of a methodical error. As far as osmotic pressure is concerned, Lillie[4] states that salts depress the osmotic pressure of neutral solutions of egg albumin in the following order

$$SO_4 > Cl > Br > I > NO_3 > CNS$$

At neutral reaction of the albumin solution the anions of a salt can have no influence on the osmotic pressure of a protein solution. Neither Lillie nor any of the other authors had measured the pH of their solutions or gels, and the Hofmeister series on osmotic pressure and swelling are the result of this methodical error, as a consequence of which these authors mistook effects due to variation of the pH for effects due to the nature of the anion. If we wish to study the effect of anions on the four physical properties of proteins—membrane potentials, osmotic pressure, swelling, and viscosity—we must use protein-acid salts, the pH of which is in the case of gelatin less than 4.7, and we must make sure that the pH of the protein solution or protein gel is not altered by the addition of salt. This can be done only by controlling the pH with the hydrogen electrode, as will be shown in the following experiments.

Membrane Potentials.

For these experiments three stock solutions all of pH 3.8 were prepared: first, solutions of gelatin chloride of pH 3.8 containing 2 gm. dry weight of originally isoelectric gelatin and 8 cc. of 0.1 N HCl in 100 cc. solution; second, M/2 solutions of different salts brought to a pH of 3.8 by the addition of HCl; and third, distilled water brought also to the pH of 3.8 by the addition of HCl. By successive dilution of the M/2 salt solutions of pH 3.8 with distilled water of pH 3.8, series of salt solutions of different degree of concentration, but all of pH 3.8, were prepared. 50 cc. of the 2 per cent solutions of gelatin chloride of pH 3.8 and 50 cc. of the salt solutions of pH 3.8 were then mixed and 1 per cent solutions of gelatin chloride in salt solutions of different concentrations, but all of pH 3.8, were obtained.

[4] Lillie, R. S., *Am. J. Physiol.*, 1907–08, xx, 127.

Collodion bags of about 50 cc. volume were filled with such solutions (closed with stoppers perforated by manometer tubes as described), and the collodion bags were submerged in beakers containing each 350 cc. of the same salt solution as that inside the collodion bags and all of pH 3.8, but without protein. The experiments lasted for 18 to 24 hours at 24°C. At that time osmotic equilibrium was reached, the osmotic pressure was read, and the membrane potentials between the protein solution inside the collodion bag and the outside aqueous solution free from protein were measured with a pair of indifferent calomel electrodes (in saturated KCl) as described.

Fig. 1 represents the effect of six different salts with monovalent anions, NaCl, NaBr, NaI, $NaNO_3$, NaCNS, and Na acetate, and one salt with divalent anion, Na_2SO_4. These salts were selected to find out whether the above mentioned Hofmeister series are real or whether the valency rule holds. The abscissæ are the concentrations of the salts and the ordinates are the observed membrane potentials in millivolts. When no salt was added, *i.e.* when the concentration was zero, the values vary within about 3 to 4 millivolts due to the limits of experimental accuracy. This variation was, of course, also found when salts were added. The curves in Fig. 1 show that the variations between the effects of the six different salts with monovalent anion are chance variations such as are observed when no salt is added, *i.e.* at concentration 0 in Fig. 1, and that if this is taken into consideration it is found that the effects of the six salts on membrane potentials lie all on one curve. The curve for Na_2SO_4 is, however, considerably lower than the curve for the six salts with monovalent anions; namely, a little less than two-thirds. The curves in Fig. 1, therefore, show that the six salts, NaCl, NaBr, NaI, $NaNO_3$, NaCNS, and Na acetate, have (within the limits of accuracy of measurements) the same effect on the membrane potentials, while the depressing effect of SO_4 is about two-thirds or still less. These experiments prove the valency rule but contradict the Hofmeister ion series.

In order to bring 100 cc. of M/2 stock solutions of the different salts to a pH of 3.8, the solutions had to contain different amounts of 0.1 N HCl, as Table I shows.

Attention is called to the enormous amount of HCl required to maintain the pH of 3.8 in the presence of M/2 Na acetate. Yet the

older colloid chemists compared the effect of Na acetate with that of NaCl without correcting for the difference of pH.

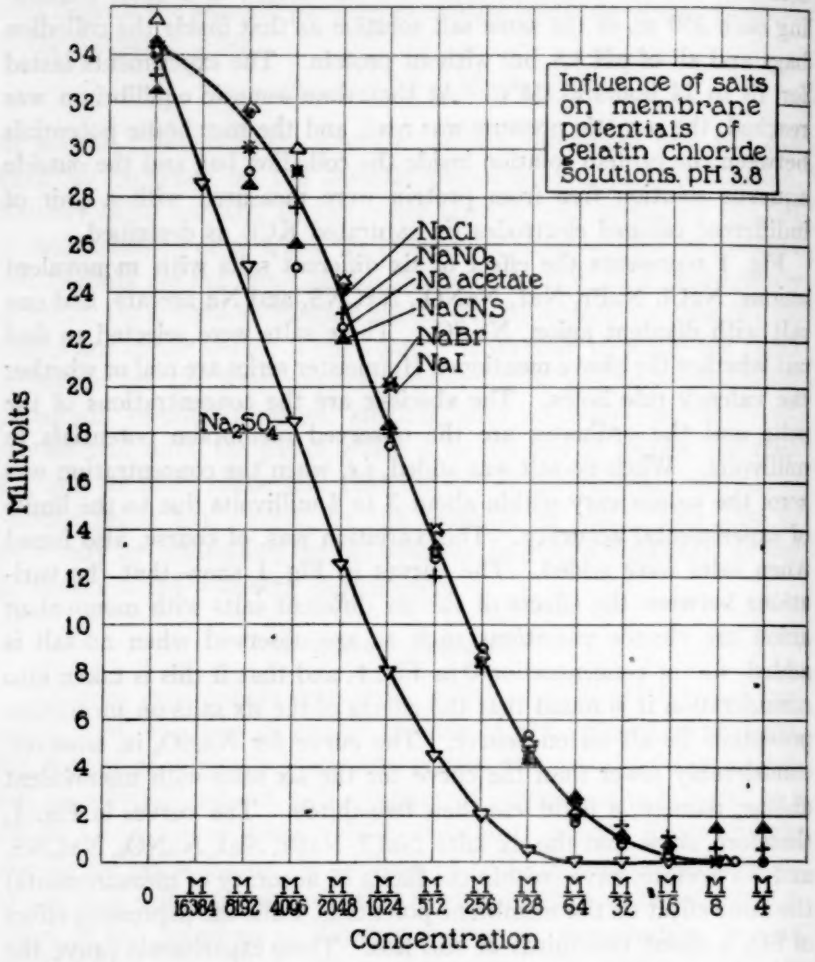

FIG. 1. All salts with monovalent anions have within the limits of experimental accuracy the same depressing effect on the membrane potentials of gelatin chloride solutions at pH 3.8, while the depressing effect of Na_2SO_4 is much greater.

This fact has to be kept in mind when the effect of buffer salts with divalent anions is examined. The following number of cc. of

0.1 N HCl (or their equivalent) was required in 100 cc. of M/2 solutions of such salts to produce a pH of 3.8.

Na_2 oxalate	55.0 cc. 0.1 N HCl
Na_2 tartrate	150.0 cc. 0.1 N HCl
Na_2 succinate	430.0 cc. 0.1 N HCl

Now in these solutions, NaCl and weak dibasic acids are formed, the latter dissociating chiefly, though not exclusively, as monobasic acids. By adding such buffer salts to a solution of gelatin chloride of pH 3.8 and maintaining the pH by adding HCl, part of the bivalent anions of the salts are transformed into monovalent anions. Hence

TABLE I.

Cc. of 0.1 N HCl in 100 cc. M/2 Solutions of Salt Required to Produce a pH of 3.8.

	0.1 N HCl required.
	cc.
NaCl	0.2
NaBr	0.6
NaI	0.3
$NaNO_3$	0.3
NaCNS	0.7
Na acetate	425.0
Na_2SO_4	1.0

the depressing effect of the three above mentioned salts should lie between that of NaCl and that of Na_2SO_4, but that of Na_2 oxalate or Na_2 tartrate nearer to Na_2SO_4 than that of Na_2 succinate. Fig. 2 shows that this is correct.

The older authors ignored the pH and compared the effect of *e.g.* Na_2 tartrate with that of NaCl, disregarding the change in pH. Their error was not one of colloid chemistry, but one of physical chemistry. When the pH of the gelatin solution is kept constant, the influence of salts on membrane potentials of a gelatin chloride solution is determined only by the valency of the anion of the salt but not by its chemical nature.

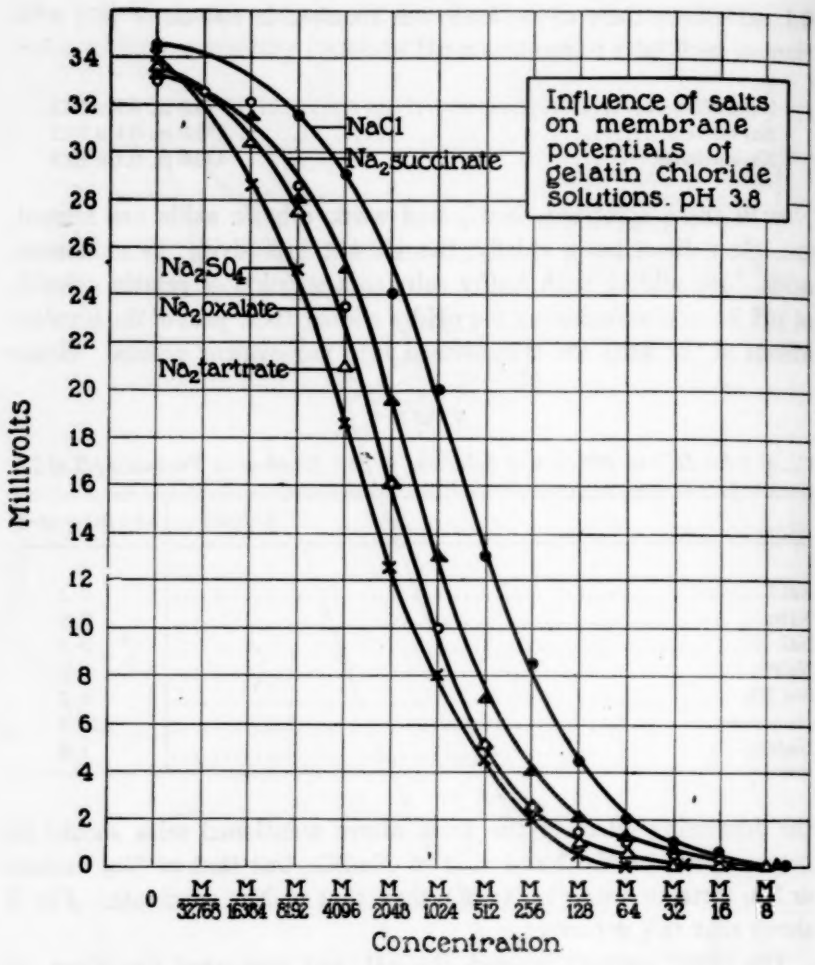

FIG. 2. Effect of strong and weak dibasic salts on the membrane potentials of gelatin chloride solutions at pH 3.8.

Osmotic Pressure.

In the preceding experiments on membrane potentials the influence of the salts on osmotic pressure of the protein solutions at pH 3.8 was also measured. Fig. 3 gives the result. The ordinates are the observed osmotic pressures in terms of a column of water. The de-

pressing effects of the six salts with monovalent anion (NaCl, NaBr, NaI, $NaNO_3$, NaCNS, Na acetate) on the osmotic pressure of the gelatin chloride solutions of pH 3.8 (containing 1 gm. dry weight of

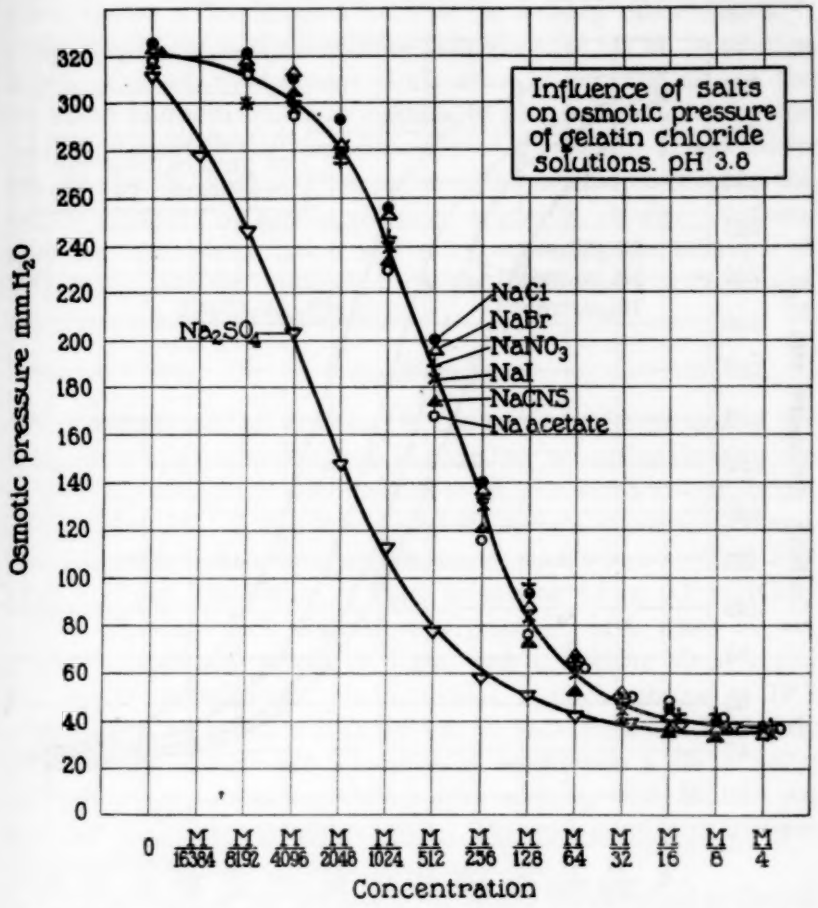

FIG. 3. All salts with monovalent anions depress the osmotic pressure of gelatin chloride solutions of pH 3.8 to the same extent (within the limits of experimental accuracy). Na_2SO_4 depresses considerably more.

originally isoelectric gelatin in 100 cc.) lie, within the limits of experimental accuracy, on one curve, which is entirely different from the curve for the effect of Na_2SO_4. The osmotic pressures are a little

over twice as high when the anion of the salt is monovalent than when it is divalent. The variations in the effects of the six salts with monovalent anion are chance variations, since they are also found when no salt is added, *i.e.* at concentration 0 in Fig. 3.

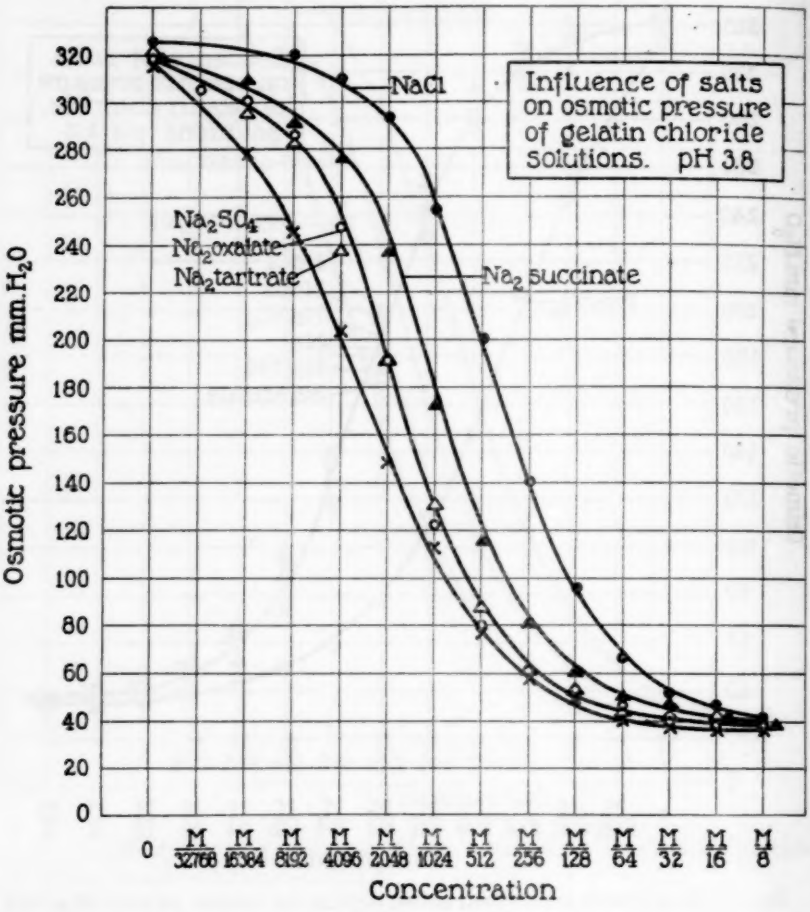

FIG. 4. Depressing effect of weak dibasic and tribasic salts on osmotic pressure of gelatin chloride solution of pH 3.8.

This shows that all the salts with monovalent anions have the same effect on the osmotic pressure when the pH is kept constant and that the Hofmeister anion series is based on error. The anion series has

to be replaced by the valency rule. This statement is supported by Fig. 4 which gives the influence of Na_2 oxalate, Na_2 tartrate, and Na_2 succinate upon the osmotic pressure. The effect of these salts lies between that of Na_2SO_4 and NaCl as the valency rule demands.

Hence we come to the conclusion that when the pH of the solution is kept constant, the influence of the anion of different salts on the osmotic pressure of a gelatin chloride solution is determined by the valency, but not by the chemical nature of the anion. It was shown in former publications that the cation of the salt has no effect on the osmotic pressure of gelatin chloride or edestin chloride solutions. Both the Hofmeister cation and anion series for the influence of salts on the osmotic pressures of protein solutions are fictitious and the consequence of a methodical error.

Swelling.

The general method described in the first part for measuring the influence of acids on swelling was also used for measuring the influence of salts on swelling. It was intended to use gels which at equilibrium had a pH of 3.8. It had been found in previous experiments that 1 gm. of powdered isoelectric gelatin has at equilibrium a pH of 3.82 when it is put into 150 cc. of water containing 4.5 cc. 0.1 N HCl.

The following stock material was prepared: first, doses of wet powdered isoelectric gelatin of 8 gm. each containing about 1 gm. dry weight of isoelectric gelatin; second, a stock solution of HCl containing 3 cc. of 0.1 N HCl per 100 cc. H_2O (since the isoelectric gel of gelatin had in such a solution at equilibrium a pH of 3.82); and third, M/2 solutions of various salts made up with HCl to the same pH as the stock solution of HCl. The stock solution of HCl was used for the dilutions of the M/2 salt solutions.

Doses of 8 gm. of the wet isoelectric gelatin (as described in Part I) were added to 150 cc. of various salt solutions made up as described, and allowed to stand for 2 hours in the solutions at 15°C. The swelling of the gelatin was then measured by weight, as described in Part I.

In the figures the abscissæ are the concentrations of salts, and the ordinates are the weights of the gelatin. Without salts the weight varied generally at the end of the experiment around 27 gm., while it was depressed by salts to about 10 gm.

It had already been shown in preceding papers[2] that when different chlorides, *e.g.* NaCl, $CaCl_2$, or $LaCl_3$, are added to a solution of gelatin chloride, the depressing effect of the three salts on osmotic pressure and membrane potentials is the same for equal concentrations of Cl ions of the salt, provided that the pH is kept constant. This agrees with the theory that the effect of salts is a consequence of membrane equilibria. It seemed of importance to see whether the same is true for the influence of salts on swelling. The influence of LiCl, NaCl, KCl, $CaCl_2$, and $LaCl_3$ was tested and the results are given in Fig. 5. The abscissæ are the molar concentrations of the Cl ions of these salts,

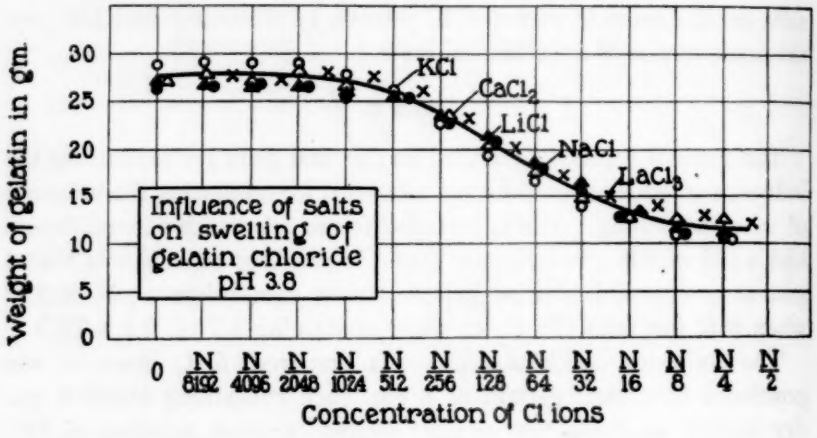

FIG. 5. All chlorides depress the swelling of a gelatin chloride gel of pH 3.8 to the same extent at the same concentration of Cl ions.

while the ordinates are the amount of swelling measured by the weight of the gelatin. It is obvious that the effects of these five salts on swelling lie all on one curve, proving that the effect of salts on swelling of gelatin chloride is determined only by the anion of the salt and that the cation has no effect whatever on the swelling of gelatin chloride.

The next fact to be ascertained was whether or not only the valency of the anion of the salt is of influence or whether the anion series generally quoted in colloidal literature is valid, according to which the swelling is a maximum in NaCNS, and a minimum in Na acetate (leaving the divalent anions out of consideration for the present).

Seven salts with monovalent anions were tried; namely, NaCl, NaBr, NaI, $NaNO_3$, NaCNS, Na acetate, and Na lactate. The results are given in Fig. 6. It is obvious that the effects of all of these seven salts lie on one curve, and that the variations are essentially the chance variations due to the limits of experimental accuracy. This is proven by the fact that the same variations are observed when the concentration of salt is zero; *i.e.*, when no salt is added. There is not the slightest indication of the Hofmeister anion series. Slight influences

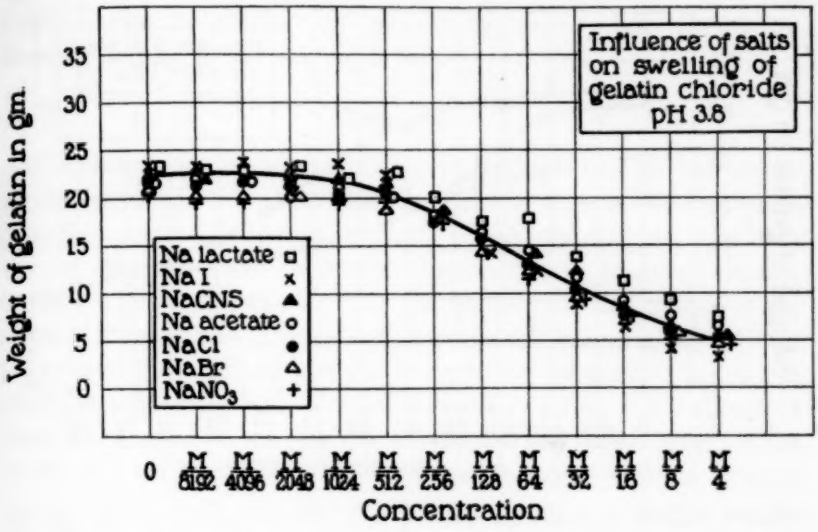

FIG. 6. All salts with monovalent anions depress the swelling of a gelatin chloride gel to the same extent (within the limits of experimental accuracy) at pH 3.8.

of the salts on the cohesion of the gel of gelatin may exist, but they are too small to play a rôle.

While salts with monovalent anions have the same depressing effect for the same concentration of anions, salts with bivalent anions have a much greater depressing effect on swelling than salts with monovalent anions. This is illustrated in Fig. 7 showing the difference in the effect of equal molar concentrations of NaCl and Na_2SO_4 on swelling. NaCl does not depress swelling in concentrations of M/1,024 or below, and the depressing effect of NaCl on the swelling of gelatin chlo-

ride of pH 3.8 commences to be noticeable at a concentration of M/512. This is true for all salts with monovalent anions as Fig. 6 shows. Na_2SO_4 begins, however, to depress at a concentration between M/4,000 and M/2,000, and the curve for the SO_4 effect drops much more rapidly to the minimum than in the case of NaCl.

The effects of salts on the viscosity of gelatin solutions have already been discussed so fully in previous publications by Loeb that it does not seem necessary to add to the facts already published.

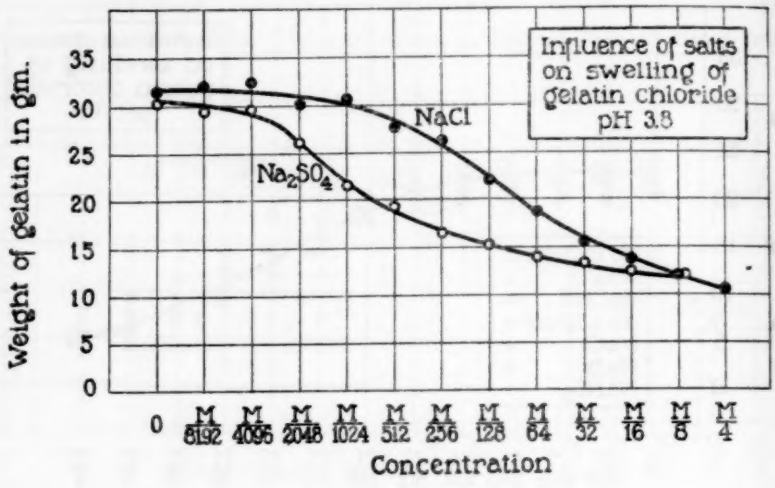

FIG. 7. Na_2SO_4 depresses the swelling of a gelatin chloride gel considerably more than NaCl.

Summarizing all the results, we can say that the membrane potentials, osmotic pressure of gelatin chloride solutions or the swelling of gelatin chloride gels, and that type of viscosity of gelatin chloride solutions which depends on the swelling of submicroscopic solid particles in the solutions are only affected by the anion but not by the cation of a salt; that all anions of the same valency have the same depressing effect on these four properties of gelatin chloride, and that the depressing effect is greater for the divalent than for monovalent anions.

The so called Hofmeister series of the effects of cations as well as of anions on these four properties are fictitious and the result of the

fact that the previous authors have failed to measure the pH of their solutions or of their gels. If this error is avoided and if the pH is kept constant, it is obvious that the Hofmeister series has to be replaced by the valency rule as was to be expected from the membrane equilibria theory of colloidal behavior. This is true only for those four properties of proteins which depend upon membrane equilibria; namely, membrane potentials, osmotic pressure, swelling, and that type of viscosity which depends on swelling. Such properties of proteins as do not depend on the Donnan equilibrium, *e.g.* solubility or cohesion, may be affected not only by the valency but also by the chemical nature of the ions of a salt.

SUMMARY.

It is shown by the older experiments by Loeb and by the experiments reported in this paper that the effect of salts on the membrane potentials, osmotic pressure, swelling of gelatin chloride, and that type of viscosity which is due to the swelling of protein particles, depends only on the valency but not on the chemical nature of the anion of the salt, and that the cation of the salt has no effect on these properties, if the pH of the protein solution or protein gel is not altered by the salt. The so called Hofmeister series of salt effects on these four properties are purely fictitious and due to the failure of the former authors to measure the hydrogen ion concentration of their protein solutions or gels and to compare the effects of salts at the same pH of the protein solution or the protein gel. These results confirm the older experiments of Loeb and together they furnish a further proof for the correctness of the idea that the influence of electrolytes on the four properties of proteins is determined by membrane equilibria. Such properties of proteins which do not depend on membrane equilibria, such as solubility or cohesion, may be affected not only by the valency but also by the chemical nature of the ions of a salt.